GENERAL CONSIDERATIONS IN MOUSE PHENOTYPING

Mice and humans share powerful genetic and biological similarities. In addition, mice are small in size, cheap to house, and have a short lifespan and large litter size, making them an excellent model for scientific investigation. Furthermore, problems of genetic and environmental heterogeneity can be minimized in the mouse, as genetic background and environmental factors can be rigorously controlled. The ability to insert or selectively delete predetermined genes in whole animals, as well as the use of genetic reference populations to study complex traits and quantitative trait loci, provide enormous power for analyzing gene function. With these successes in mouse genetics, the critical factor in the use of phenogenomic strategies is the need for an efficient and vigilant process for mouse phenotyping. It is now recognized that the phenotype of a mouse (behavioral, metabolic, and so on) is highly dependent on genetic, developmental, and environmental factors. All phenotyping experiments must begin with careful consideration of these factors, which can influence all variables and bias the resulting phenotype.

Uses of Forward and Reverse Genetics in Mice to Study Gene Function

The purpose of this chapter is to bridge the gap between molecular biology and physiology so that the novice mouse user has the required vocabulary and background information to understand the molecular origin of the mouse to be phenotyped (also see Argmann et al., 2005). This unit summarizes the diverse genetic strategies used in the mouse, and serves as a prelude to the detailed phenotyping protocols that follow. Protocols for carrying out the genetic manipulations themselves are described elsewhere in this manual, and appropriate units are cross-referenced here.

It is becoming common practice for genetically engineered mouse models (GEMMs) to be generated in collaboration with core facilities having technical expertise in this field. Furthermore, many GEMMs or genetic reference populations (GRPs) can be readily purchased from commercial suppliers. There are a number of ongoing attempts to facilitate the accessibility of available mouse strains, including an international resource that is presently being consolidated in the form of a searchable online database (*http://www.informatics.jax.org/imsr/index.jsp*). This database will regroup all mouse strains and stocks available worldwide, including inbred, mutant, and genetically engineered strains. For additional mouse-related Web sites, see Internet Resources.

REVERSE GENETICS

Reverse genetics is a gene-driven approach that deduces, from directed mutations, the full range of phenotypes controlled by each gene. This strategy relies on engineering the mouse genome through adding, subtracting, and replacing genes to control gene activity temporally, spatially, and reversibly. Three reverse genetics approaches are described below.

Transgenics

The production of transgenic mice (*UNIT 23.1*) is a gain-of-function approach to manipulation of the mouse genome (Clarke, 2002; Nagy et al., 2003; Hedrich, 2004). Transgenic mice can be designed for numerous purposes including: (1) identification and characterization of specific regulatory sequences; (2) ubiquitous or tissue-specific overexpression of a transgene; and (3) cell lineage ablation achieved by coupling a tissue-specific promoter to a sequence encoding a toxin which kills cells that express it. Transgenic mice are typically generated by microinjection of the transgene construct into the male pronucleus of fertilized oocytes, which are then

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reimplanted into the oviducts of foster females (*UNIT 23.9*). Expression of the transgene is dependent on its site of integration, with individual insertion sites typically containing multiple copies of the transgene. It is important to keep in mind that transgene copy number and expression levels of the transgene do not necessarily correlate. When chromosomal integration occurs in the fertilized oocyte, the developing animal will be fully transgenic, since all nucleated cells in the animal will contain the transgene; however, if integration occurs later, at a postzygotic stage, the animal will be a mosaic, with some cells containing the transgene and others lacking it. Founders are identified through analysis of tail biopsies, and copy number and transgene integrity are then evaluated. To establish a transgenic line, a founder is bred to mice of a defined genetic background and offspring are characterized for expression of the transgene. Generally at least two founders should be maintained for experiments. Due to the nature of random integration, no two transgenic lines will be identical. Details on the identification and analysis of founders and the establishment of transgenic lines can be found in *UNIT 23.9*.

Transgenics carrying large segments flanking a gene of interest isolated as bacterial artificial chromosomes (BACs) have been used to minimize the abovementioned variation due to the effects of different integration sites (*UNIT 23.1*). The versatility of this strategy has been significantly increased by the development of homologous recombination–based strategies to mutagenize and manipulate the BAC constructs (*UNITS 1.16 & 23.11*) and the use of regulated transgenics (e.g., with tetracycline; *UNIT 23.12*). In the GENSAT project (*http://www.ncbi.nlm.nih.gov/projects/gensat*; Gong et al., 2003), for example, an atlas of gene expression in the mouse central nervous system is based on large numbers of BAC transgenics in which the protein-coding sequences of genes expressed in the brain are replaced by an EGFP reporter.

Knockouts

Gene knockout or ablation (*UNIT 23.1*) is a powerful strategy to define the range of phenotypes associated with the loss of function of a gene of interest. The reverse genetic strategy of one gene–one protein–one phenotype can often aid in elaborating complex "wiring" diagrams and placing genes into biochemical pathways such as those defined by environmental stimuli, cell surface receptors, downstream targets, and changes in gene expression. There are a number of potential problems with full-knockout GEMMs, however, particularly when the phenotypes of homozygous null mutants are extreme (e.g., when they result in embryonic or perinatal lethality).

This drawback has been overcome through the increasing use of conditional GEMMs, whose genomes are subject to temporal and spatial controls (*UNIT 23.1*). To generate conditional knockout mice, a conventional gene-targeting strategy is used to generate homologous recombinants, whereby parts of the gene of interest are flanked by site-specific recombination sequences such that the gene retains normal function in the embryonic stem (ES) cells and offspring mice. The most commonly used site-specific recombination system is the Cre-*loxP* system from P1 bacteriophage (Branda and Dymecki, 2004). Gene-targeting constructs are generated so that *loxP* elements flank the region of the genome to be deleted. Following germline transmission, mice that carry alleles flanked by *loxP* ("floxed" alleles) are subsequently bred with a tissue-specific Cre transgenic mouse. The resulting bigenic mice carry both floxed alleles and a Cre transgene (which can be tissue specific). The Cre recombinase mediates recombination between two *loxP* sequences, thereby excising the sequence between the two *loxP* sites and hence creating a gene deletion. An added sophistication to such spatially controlled mutagenesis is the inclusion of temporal control, which can be achieved by using ligand-activated chimeric recombinases (Metzger and Chambon, 2001). These are usually chimeric proteins in which the recombinase is fused with the ligand-binding domain of a nuclear receptor, such as the estrogen or progesterone receptor, enabling gene ablation at any time by administration of the corresponding nuclear receptor ligand, e.g., tamoxifen or RU-486 (Metzger and Chambon, 2001). The expression of a transgene can also be controlled in a temporal fashion through the use of either hormonally (e.g., ecdysone) or pharmacologically (e.g., tetracycline) inducible gene-expression systems.

Gene Trapping

Gene trapping is a mutagenesis strategy that involves the transfection of ES cells with a gene-trap vector, which is based on the use of a reporter and/or selection marker to identify a trapping event. One strategy involves a promoter trap, which carries a promoterless reporter gene and a selection marker gene under the control of its own promoter. In this instance, the reporter gene is transcribed only

when the construct is correctly inserted in the promoter region of an endogenous gene. Alternatively, there are polyadenylation trap strategies, in which a constitutive promoter drives the transcription of the selection markers. Because the polyadenylation-trap vector lacks a poly(A) signal, the transcript is only stabilized as long as the gene-trap vector is integrated in the correct orientation and captures the poly(A) signal of the trapped gene. Once a library of "trapped" ES cells has been selected, insertion points are precisely determined for each line by sequencing the region neighboring the gene-trap vector (Koutnikova et al., 2003; Hedrich, 2004).

Since gene-trapping mutagenesis techniques are relatively high-throughput strategies, it is expected that they will greatly facilitate functional gene annotation. For this reason, several consortia plan to carry out functional annotation of the entire mouse genome via systematic mouse genome mutagenesis using gene-trapping as their primary strategy (Austin et al., 2004; Auwerx et al., 2004). One disadvantage associated with gene-trapping is that the impact of the mutation on protein function must be carefully determined.

FORWARD GENETICS

In contrast to the reverse genetic approach, forward genetics is centered on phenotype-driven gene discovery. The approach consists of identifying a phenotype of interest in mutant mice derived by chemical mutagenesis, radiation mutagenesis, or even spontaneous mutation, and then determining the mutation responsible for the phenoytpe. Prior to the introduction of technologies for manipulating the mouse genome, this was the only genetic strategy available. Continued enthusiasm for forward genetics over specifically targeted strategies is due to the random nature of the mutations, which cover the full gamut from point mutations to insertions, deletions, and rearrangements, and thus produce a full spectrum of changes to protein structure and function.

Many of the mutant mouse models used to date in forward genetic strategies have arisen from spontaneous single-gene mutagenesis events that produced striking phenotypes. The most notable examples in the metabolic field are mutations in leptin (*Lep^{ob}*; Zhang et al., 1994), leptin receptor (*Lepr^{db}*; Chen et al., 1996), and tubby (*Tubby*; Noben-Trauth et al., 1996) genes. Human homologs of many murine obesity genes have a confirmed association with monogenic obesity in man and have helped advance understanding of the molecular pathogenesis of obesity and its complications.

Mutations can be generated by chemical means using chlorambucil or ethylnitrosourea (ENU), and also by radiation (X-ray) mutagenesis of sperm or ES cells. ENU is generally the mutagen of choice (O'Brien and Frankel, 2003; Hedrich, 2004) and is an alkylating agent that can transfer its ethyl group to oxygen or nitrogen atoms at a number of reactive sites in DNA bases. This can cause mispairing of the two strands of DNA and consequently base-pair substitutions. ENU generates point mutations in DNA at a rate of $\sim 10^{-5}$, such that $\sim$1/1000 of the offspring carry a mutation in a given gene. Although the generation of mutants is fast with ENU, the success of this technique depends on the implementation of efficient, robust, and validated high-throughput phenotyping resources for screening the progeny of treated mice for obvious phenotypic abnormalities. One major obstacle is that the mutations occur essentially at random, and the identification of the structural change(s) in the DNA generally requires a laborious breeding strategy combined with a positional cloning approach. This task remains timely and costly, as every mutant must be back-crossed to other inbred strains to identify markers that cosegregate with the mutation.

POLYGENIC DISEASES AND GENETIC REFERENCE PANELS

Currently, there is increased focus on understanding the pathogenesis of complex diseases such as cancer, neurodegenerative disorders, and the metabolic syndrome. Monogenic GEMMs or randomly mutagenized mice that focus on an isolated genetic locus in a fixed background cannot model polygenic diseases inherited in a non-Mendelian fashion. Moreover, environmental factors can affect the manifestations of the genotype, resulting in incomplete penetrance, or mimic the contribution, resulting in phenocopies. Thus, it is desirable to have mouse models that mimic the genetic structure of human populations. Oligogenic mouse models, which are engineered with mutations in two or more genes, can be valuable in such a context and have illustrated many of the genetic principles underpinning complex diseases. Some key principles to keep in mind include: (1) that a major predisposing allele can have a modest effect alone, but play a major role in the context of a predisposing background, (2) that mutations in

different unrelated genes can yield the same phenotype (Nandi et al., 2004), and (3) that the interaction among some genetic loci can create strong positive or negative (protective) effects in the predisposition to a disease. Other genetic principles such as those underlying intertissue communication and cross-talk have been elucidated through the use of spatially and temporally controlled GEMMs.

An increasingly popular tool for the study of mouse physiology that complements the forward and reverse genetic strategies with a polygenic approach is the mapping of quantitative trait loci (QTLs) in inbred mice. Genetically homogeneous inbred strains have an inherent wealth of variation due to past spontaneous mutation events, and they can reveal subtle variation affecting many traits of biomedical interest (Paigen, 2003). The Complex Trait Consortium was formed to systematically exploit large-scale resources with the aim of partitioning strain differences in hundreds of quantitative traits to sets of biologically related genes or loci, and thereby identify genomic regions and genes associated with these traits (Threadgill et al., 2002). However, mapping QTLs based solely on the parental strain phenotypes is not an effective strategy, since many genetic contributors to complex diseases will be missed (Li et al., 2005). This is because the power and accuracy in detecting a QTL depends on multiple factors, including the genetic diversity of the parents, the density of the markers, the trait heritability, and the size of the cross. In any single or double cross, recombination events and mapping resolution are thus limited, as only loci that show allelic variation between two strains can be detected.

Two recent advances improve collection of data from resource-intensive breeding crosses. The first is the use of multiple crosses (two or more single crosses), which improves the power and resolution (Li et al., 2005). With single intercrosses, it is difficult to distinguish multiple tightly linked QTL from a single QTL with a large effect, and the QTL intervals defined are generally quite large, typically 20 to 40 cM. With multiple crosses, a computational meta-cross analysis can be performed. When the same QTL is found in more than one cross, the data can be combined from the crosses for the chromosome on which the QTL is found. If the QTL from multiple crosses are caused by the same gene, the impact of combining the cross data is equivalent to that of adding more recombination events in the QTL region (e.g. by additional mice in a cross). This will narrow the QTL region and increase the power for resolving the QTL. For example, a QTL for HDL cholesterol was found on chromosome 4 in four different crosses, and, by combining the data from these crosses, the QTL was narrowed from 30 cM to 10 cM. (Li et al., 2005). The second is the generation of several large genetic reference panels (GRPs) of recombinant inbred (RI) mouse lines that have a higher rate of polymorphism between strains. GRPs are a set of genetically well-defined strains derived by crossing two different highly inbred progenitor strains and then inbreeding random pairs of the F2 generation (>20 generations) to produce a series of RI strains that represent an ~50:50 genetic mixture of the progenitor strains (Churchill et al., 2004; Peirce et al., 2004; Fig. 29A.1.1). Thus, the strains that make up a GRP are not mutants or engineered mice, but are normal mouse lines with a level of variation that more closely resembles that seen among humans. GRPs are also efficient because they are a renewable resource; genotyping only needs to be performed once, and genetically identical animals can then be distributed to many researchers (Churchill et al., 2004).

With recent advances in functional genomic technologies, such as the widespread use of high-density microarrays, the power to decipher complex molecular networks underlying the multifactorial nature of common disorders has been markedly increased. This is best illustrated when applied to the analysis of GRPs. The approach initially applied in yeast involves carrying out genome-wide genetic analyses of gene expression data, which are then considered to be a quantitative trait and are correlated with microsatellite markers of DNA variation (Damerval et al., 1994; Jansen and Nap, 2001; Brem et al., 2002; Schadt et al., 2003; Bystrykh et al., 2005; Chesler et al., 2005). Those genetic regions (loci) that account for variation in the levels of gene expression, termed expression quantitative trait loci (eQTL), reveal previously unknown gene-gene interactions and biochemical pathways. Compared to classical clinical quantitative trait loci (cQTL), which often include gross clinical measurements that are far removed from their causative biological processes, such eQTL approaches offer an additional level of sophistication, especially when combined with the analysis of disease traits such as cQTL. Many "omic" disciplines such as proteomics, metabolomics, and pharmacogenomics have followed in the

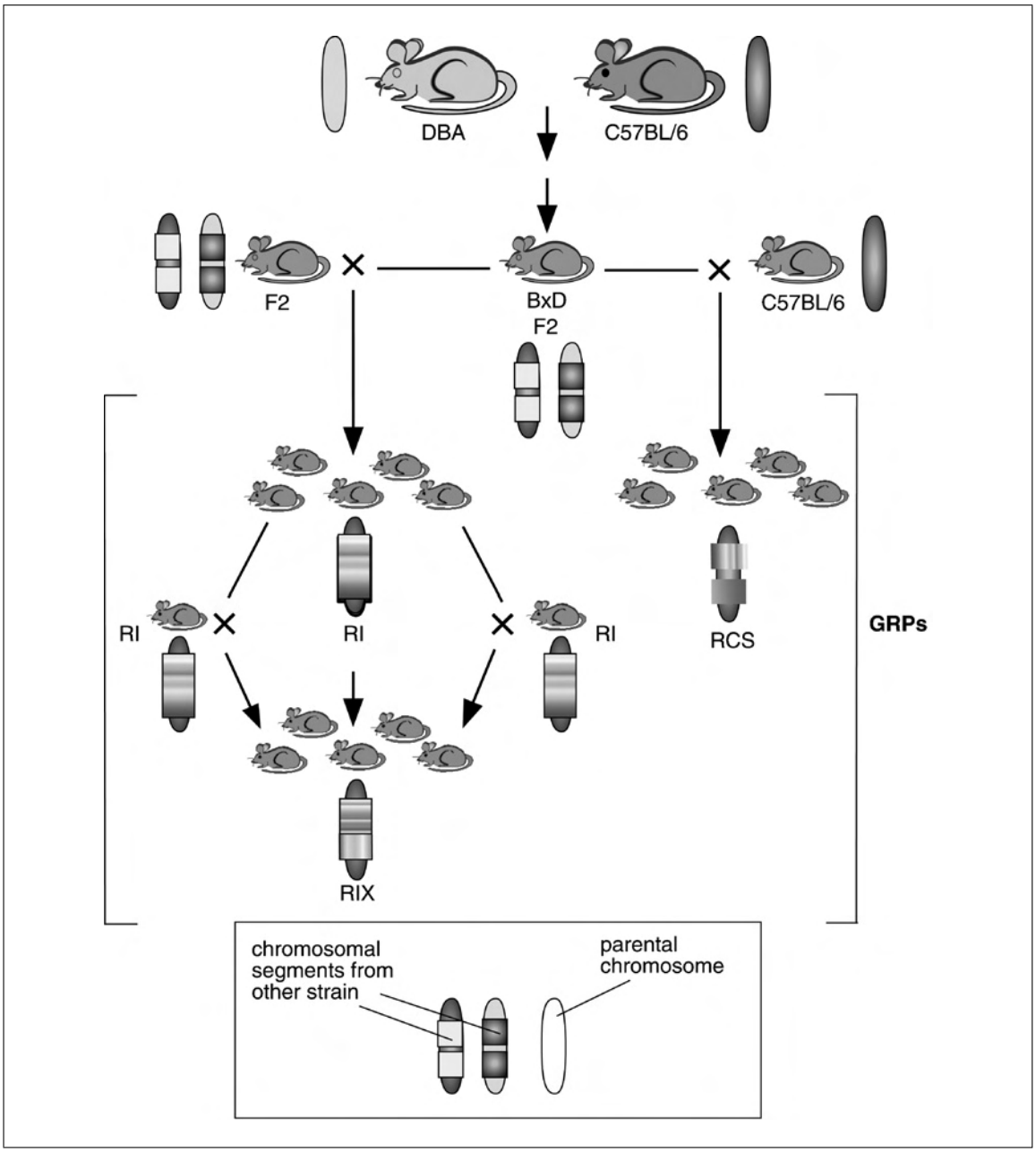

Figure 29A.1.1 GRPs can be defined as a set of genetically well-defined strains derived by systematical inbreeding, for at least 20 generations, from a single cross of two distinct inbred strains. GRPs represent an ~50:50 genetic mixture of the progenitor strains (Churchill et al., 2004; Peirce et al., 2004). Thus, the strains that make up a GRP are not mutants or engineered mice; they are normal mouse lines that have a level of variation more similar to that among humans. Approaches based on recombinant inbred (RI) strains are continually evolving to offer the variability, power, or resolution necessary for a general-purpose mapping tool, since at present there are too few GRPs with limited QTL power. Some of the sophistications include the use of recombinant congenic strains (RCS), which are variations of RI strains, whereby two parental inbred strains are initially crossed, but the resulting F1 hybrid progeny are then back-crossed with one of the parental strains prior to sib-pair matings. Instead of the mix of progenitor strain genomes approaching 50:50, the genome of RCS is representative mainly of one parent. The limitations of the current RI lines have also spurred an effort to create as many as 1000 new RI lines of mice within 7 years with the hope of covering much of the genetic variation present in natural populations. It will then be possible to generate an almost unlimited combinatorial diversity using the F1 progeny of these RI strains (RIX; Churchill et al., 2004).

footsteps of these genomic approaches. Application of these strategies to the analysis of either GEMMs or GRPs holds great promise and has clearly diversified the menu of mouse models to study human metabolic diseases.

It is becoming apparent that the best strategy for modeling complex diseases is to effectively combine data generated using relatively simple forward and reverse genetic strategies (whereby scientists drive gene elimination to precipitate disease) with global analyses of complex polygenic GRPs (where nature drives genetic variation to recreate disease). This combined strategy allows the benefits of the clear-cut results of single gene perturbations (e.g., knockout mice) to be merged with those conferred by the subtle alterations from innumerable allelic variants (GRPs).

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INTERNET RESOURCES

http://genex.hgu.mrc.ac.uk

Edinburgh Mouse Atlas (emap) and gene expression database (emage).

http://www.ensembl.org/Mus_musculus

Ensembl Mouse Genome Server.

http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase/

ENU Mutagenesis Program.

http://www.eumorphia.org

EU Eumorphia program.

http://www.emma.rm.cnr.it

European Mouse Mutant Archive (EMMA). Transgenic mouse repository.

http://www.informatics.jax.org

Festing's Inbred Strain Characteristics.

http://www.eulep.org/Necropsy_of_the_Mouse

Gross necropsy and tissue fixation.

http://www-mci.u-strasbg.fr/

Institut Clinique de la Souris (ICS) or Mouse Clinical Institute.

http://www.informatics.jax.org/imsr/index.jsp

International Mouse Strain Resource. Aims to list all publicly available mouse strains.

http://www.informatics.jax.org

Mouse Genetics: Concepts and Applications. L.M. Sliver's Mouse Genetics Book online.

http://www.informatics.jax.org/mgihome/nomen/table.shtml

Mouse Genome Informatics.

http://www.biomednet.com/db/mkmd

Mouse Knockout and Mutation Database. Searchable database for phenotypic information related to GEMMs.

http://www.mousephysio.com/

MousePhysio.

http://www.mymouse.org

Mymouse.org. Aims to facilitate international collaborative research among mouse users.

http://eulep.anat.cam.ac.uk/

Pathbase.

http://www.eumorphia.org/EMPReSS/

Standardized Operating Procedures. Covers phenotyping to necropsy.

http://tbase.jax.org

TBASE. Database for transgenic and knockout mice.

http://www.hgmp.mrc.ac.uk/DHMHD/dysmorph.html

The Dysmorphometric Human-Mouse Homology Database.

http://www.genenetwork.org/

The Gene Network. Bioinformatic toolkit for genetic reference populations (GRPs).

http://www.jax.org

The Jackson Laboratory. Mouse vendor and genetic resource center.

http://www.nih.gov/science/models/mouse/index.html

Trans-NIH Mouse Initiative.

http://www.jax.org/phenome

U.S. Mouse Phenome Project.

http://www.rodentia.com/wmc

Whole Mouse Catalog. An information Web site.

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Minimizing Variation Due to Genotype and Environment

The observation of different phenotypes in mouse strains that have undergone identical genetic manipulations, pharmacological interventions, and/or environmental stresses is not uncommon. This is because linking genotype directly to phenotype is often complicated by the fact that the genetic background of the mouse greatly impacts its phenotype. Thus, it is important that genetic factors such as strain background be considered in the context of any phenotypic studies, as they have an influence on almost all variables. Results obtained in one genetic background do not necessarily translate into another background. While this is perhaps the most obvious caveat that may affect the outcome of a phenotype, it is by certainly not the only one. In reality, there is a plethora of confounding factors (Table 29A.2.1), including genetic, developmental, environmental, and technical.

GENETIC BACKGROUND

Discrepancies in the outcomes of phenotyping tests reported by different laboratories evaluating the same genotype have provided a major driving force in addressing the need for standardization. For example, parallel testing of mouse behavior carried out by three independent laboratories on mice produced from the same litters and breeding stocks, in similar setups with identical protocols, led to significantly different results (Crabbe et al., 1999). In this experiment, the genotype accounted for a significant proportion of the total variability in behavior, and historically documented strain differences were reproduced across all laboratories. For example, C57BL/6J mice are known to prefer ethanol, while DBA/2J mice avoid it. Similarly, C57BL/6J are good learners whereas DBA/2J are poor learners in hippocampus-dependent tasks (Upchurch and Wehner, 1988; Stiedl et al., 1999). These types of strain differences may significantly affect the outcome of certain phenotypic analyses.

Strain type must also be carefully chosen for metabolic/endocrine studies (addressed in Section 29B), as different strains are known to respond differently to metabolic challenges. For example, C57BL/6J mice are more susceptible to diet-induced diabetes and atherosclerosis compared to other strains (Paigen et al., 1985; Schreyer et al., 1998). C57BL/6J mice have low plasma cholesterol and low plasma triglycerides (TG), but high glucose levels in contrast to C3H mice, which generally have low blood pressure and high plasma cholesterol, TG, and glucose. Inbred 129 strains have a very low glucose production rate but high plasma cholesterol and low TG levels (Burgess et al., 2005). In addition, 129 strains are generally less active (Seburn, 2001) and are poor breeders. For additional information on mouse strain characteristics, readers should refer to the Mouse Phenome Database at *http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home* and to

Table 29A.2.1 Genetic, Environmental, and Experimental Factors that Influence Phenotypic Analysis

	Possible confounding factors
Animal	Age, gender, ovarian cycle, health status, treatment with drugs
Development	Intrauterine position effect, intrauterine feeding, maternal behavior
Genetics	Genetic background, gender, compensation, pleiotropy
Environment	Housing conditions, handling, stress, social hierarchy
Experimenter	Level of experience, same or different experimenters
Experimental design	Construction, standardization, illumination, time of testing (e.g., circadian rhythm)
Data acquisition and analysis	Calibration/thresholds, automated versus observed, experimenter bias, normalization, statistics

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Michael Festing's index of inbred strains at *http://www.informatics.jax.org/external/festing/rat/STRAINS.shtml*.

Since C57BL/6J mice have a good breeding reputation, they are frequently the preferred genetic background for a large number of congenic strains, covering both polymorphic and mutant loci. However, due to the preferred use of 129 embryonic stem (ES) cells for gene targeting and gene trapping, a large number of studies have been performed in gene-targeted mice on a non-homogeneous genetic background. Yet the genetic background of the ES cells used to generate mutant mice has been proven to influence their phenotype. If the background strain of the phenotyped mouse is different from the one used to generate the knockout, the mice must be back-crossed for at least nine generations to avoid confounding factors contributed by flanking donor chromosomal DNA (Banbury Conference on Genetic Background in Mice, 1997). Alternatively, flanking donor chromosomal DNA can be monitored by using a marker-assisted selection protocol, enabling a quicker production of congenic strains compared to traditional backcrosses (Collins et al., 2003). Overall, these strain-specific differences show why it is very important to select the most appropriate strain for a particular study.

ENVIRONMENT

The environment is also critical in the outcome of phenotypic testing and must be carefully controlled to compare results from different laboratories. In one study, mice tested in a laboratory in Edmonton were on average more active than mice of the same genetic background tested in Albany and Portland (Crabbe et al., 1999). Whereas some of environmental factors have become experimental tools with which to challenge animals (e.g., diet), many others have become standard "housekeeping" parameters, whose order and consistency must be maintained. It is vital that the potential variables in phenotypic testing be minimized or carefully noted, so that only gene-elicited, environmentally defined mutant phenotypes are measured with similar outcomes wherever and whenever testing takes place.

Typical housekeeping items include animal housing and handling conditions such as the number of animals per cage (housing density), diurnal rhythm, length of fasting, blood collection procedure (retro-orbital or tail puncture, with or without anesthesia), diet, and age and gender of the mice (Champy et al., 2004). Cage housing density is an important contributor to stress and significantly impacts many metabolic variables. Group-housed male mice behave differently than singly housed males with respect to territorial behavior and the formation of social hierarchies (Würbel, 2001). Similarly, housing conditions can influence many metabolic parameters. Glucose levels are significantly elevated when animals are caged in pairs as compared to when they are housed in groups of four, in part due to the increased stress and reduced competition for food intake between mice housed in pairs (Champy et al., 2004). Finer housekeeping details, such as the timing of blood collection, should also be critically controlled since metabolic variables are affected by activity and food intake, which is higher at night, and hormonal variations, which show marked nychthemeral (24-hr) rhythms (Champy et al., 2004).

Behaviors can also be affected by sex and age. The estrus status affects both emotionality and cognition in mice, and DBA/2J mice are known to become deaf by adulthood (Willot et al., 1995; Wotjak, 2004, and references therein). In a recent study involving a biochemical screen for thirteen analytes pertaining to lipoprotein metabolism, electrolyte balance, and organ function in C57BL/6J mice, twelve of the analytes were affected by the age and gender of the mice tested (Zhou and Hansson, 2004).

Developmental factors also contribute to the "individuality" and/or phenotype of the mouse, or to differences that occur despite seemingly identical genetics or environmental conditions (Lathe, 2004). Activation of the immune system during gestation, intrauterine factors (position of the embryo, nutrient and endocrine exchanges, and so on), and maternal behavior (grooming and licking) have all been shown to be determinants of behavior of adult offspring. For example, mice whose mothers displayed greater nursing activity showed a higher resistance to the deteriorating effects of stress in adulthood (Lathe, 2004, and references therein). These factors, despite being beyond standardization, are nonetheless important. In the study by Crabbe et al. (1999), for example, where genetic factors as well as laboratory environments were essentially similar, these effects may offer further explanations.

CONCLUSION

It is surprising that the impact of these important confounding factors on phenotyping has only recently been appreciated. In response to these observations, major

systematic phenotyping efforts, such as the European Union's Eumorphia program (*http://www.eumorphia.org/EMPReSS*), have been designed to standardize and validate phenotyping tests, linking them to standardized operating procedures (SOPs; Green et al., 2005). Quality standards and management, not only in industry but also in academic research, should ensure that SOPs and validated test paradigms guarantee highly reproducible results within one laboratory and between different research institutions. Obviously, standardization is limited by the confounding influences of the experimenter and the different resources provided by research institutions. Nonetheless, most of the confounding variables described above can be controlled in any given laboratory by following these guidelines.

1. Reduce genetic variation by using congenic mouse lines.
2. Reduce environmental variation through careful housekeeping.
3. Use mice of the same age and gender.
4. Use standardized operating procedures (SOPs).
5. Be conscientious and minimize sources of experimenter variation.
6. Repeat the experiment several times to ensure confidence in the data.

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Collection of Blood and Plasma from the Mouse

BASIC PROTOCOL

When working with mice, venous blood is commonly collected from the retro-orbital plexuses or the tail vein, or by cardiac puncture (Hedrich, 2004). The choice of method will depend on the quantity of blood required, the frequency of the blood draw, the skill of the blood drawer, and local animal handling guidelines. In the authors' experience, blood collection via the tail vein is the easiest method and provides sufficient material for most analyses, including lipid analysis. Furthermore, this method is used for blood sampling during numerous types of dynamic hormone tests, such as glucose tolerance tests.

In the tail of the mouse, there are three veins and one artery (Fig. 29A.3.1). As the lateral caudal veins are thicker than the dorsal caudal vein, the former are generally better for collecting large amounts of blood. The artery can also be used; however, bleeding time is longer. In general, blood is collected from awake and unrestrained mice; in this case, the procedure is easier with two persons. Alternatively, a restraining device can be employed and the blood draw can be performed by one person.

Although sometimes used interchangeably, serum and plasma are qualitatively different. In plasma, only the cells have been removed by centrifugation. In serum, the bulk of the fibrinogen has been removed by conversion into a fibrin clot together with the platelets, which have been physically bound in the fibrin matrix or activated to form aggregates or both. Since varying amounts of other proteins can be removed in the fibrin clot through interactions, and since the clotting process can induce cellular elements to secrete certain components, the quantities of various analytes (e.g., potassium) can be different in serum versus plasma. Since plasma is prepared in the presence of an anticoagulant such as EDTA, coagulation does not occur and fibrinogen is not consumed. In general, serum or plasma samples can be used for most analytes, including basic biochemistry and lipid analysis. For lipoprotein profiles, however, plasma is generally used. The researcher

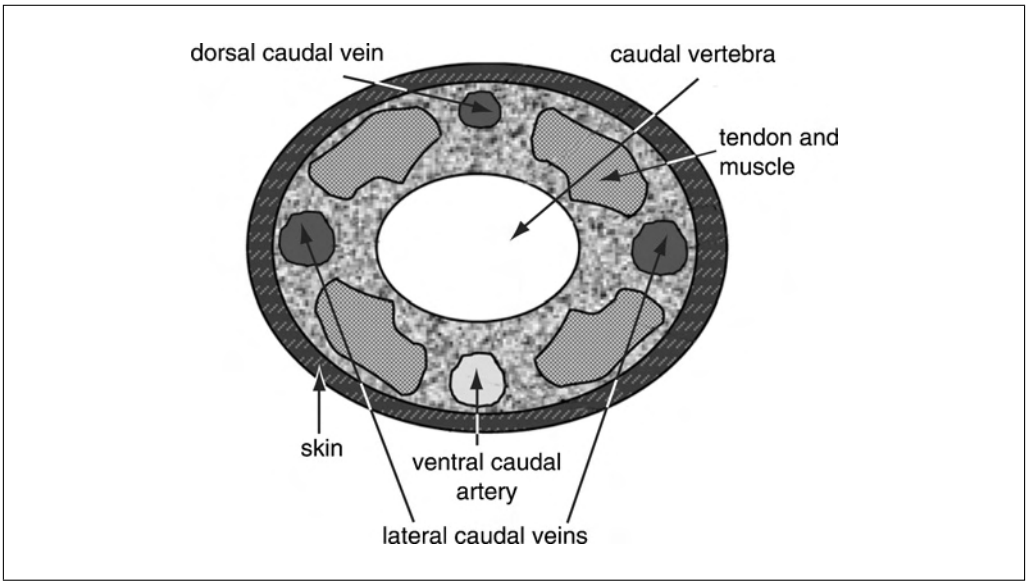

Figure 29A.3.1 A schematic drawing of a transverse section of a mouse tail showing the location of the veins and artery. The lateral caudal veins are used for venous blood collection.

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should always verify for each analyte the limitations of the experimental protocols, and should use the same processing procedure when making comparisons.

NOTE: For a more complete coverage of mouse handling (e.g., the use of restraining devices) and blood collection, refer to Hoff (2000) and Coligan et al. (2006).

Materials

Mouse
Hematocrit sealing compound (e.g., *http://www.vitrex.dk*)
Alcohol-soaked cotton pads
Sterile gauze
Surgical blade, no. 10 or 11
Microhematocrit capillary tubes (with or without Na-heparin or EDTA, depending on analyte and plasma versus serum)
5-ml polypropylene centrifuge tubes
Refrigerated centrifuge
100-μl Hamilton syringe

1. Place the mouse on the grid of a clean cage. Clean the tail with an alcohol-soaked cotton pad and then wipe it dry with sterile gauze.
2. Grip the end of the mouse's tail in one hand in such a way that the section of the tail to be cut (~1 to 2 cm from tip of tail) is held steady while the mouse is moving about.
3. Using the other hand, locate the veins running on one side of the tail.

   In general, if this procedure is performed on white or light-colored mice, the vein can be located visually. In black or dark-colored mice, this is more difficult and requires knowing the location of the vein.
4. Using a surgical blade held at a 90° angle with respect to the long axis of the tail, make a small cut on the side of the tail near the end.

   Blood should immediately flow if the cut has been made in the right location. Care should be taken not to cut too deeply, as the tail cartilage can be affected. To improve the blood flow, the tail vein can be prewarmed by placing it in water at 40°C. A more practical and common alternative is to massage the length of the mouse's tail, as this will promote blood flow. However, for hematological analyses, massaging the tail is not recommended as this will result in leukocytosis, which increases the white blood cell count.
5. While holding and/or massaging the tail, touch a glass capillary tube to the blood droplets accumulating on the mouse's tail and allow the blood to enter the capillary tube by capillary action. To promote entry of blood into the tube via gravity, lower the opposite end of the tube so that it is below the blood droplet.

   In the absence of a restraining device and with two persons performing blood collection, steps 1 to 4 should be performed by one person and the capillary tube should be manipulated by the other.

   Using this procedure, at least two microhematocrit capillary tubes can be filled with blood, which corresponds to ~200 μl of blood from an average-sized mouse.

   For plasma, use sodium-heparinized or EDTA-containing tubes to prevent coagulation. For serum, use an untreated tube, as serum is collected in the absence of an anticoagulant.
6. Plug the capillary tubes using hematocrit sealing compound and place them in a 5-ml plastic tube on ice.
7. After blood collection, compress the cut with sterile gauze until the bleeding stops.

8. Return the mouse to its cage and monitor the animal for signs of anemia, including rapid breathing, muscle weakness, and paling of mucous membranes. Observe the site of blood collection daily for infection or irritation. When large quantities of blood are drawn, allow at least 2 weeks for recovery prior to performing another blood draw.

9. To collect plasma, centrifuge the 5-ml tube (containing the hematocrit tubes) 15 min at 3000 rpm (1500 × *g*), 4°C.

   The blood generally separates into three phases, with the bottom phase consisting of red blood cells, the top phase consisting of serum, and an interphase (referred to as the buffy coat) generally resembling a thin white layer containing mainly immune-type cells.

   For serum collection, keep blood at room temperature for 1 hr in the untreated collection tubes, then centrifuge as described above.

10. Collect the plasma (or serum) from the top with a Hamilton syringe, leaving behind the buffy coat and red blood cells.

    The plasma (or serum) is ready for analysis. If necessary, store at −20°C or −80°C.

COMMENTARY

Background Information

Cardiac puncture and orbital eye bleed generally yield large quantities of blood (∼300 μl per draw) as compared to tail vein sampling (∼150 μl per draw), assuming that an average-sized mouse is used and it is not overbled. However, these former techniques may require the sacrifice of the animal (where blood draw quantity can be even higher, ∼900 μl), may blind the animal, and require a great deal of skill. Thus, the tail vein method provides a less invasive procedure that allows repeated blood draws from the same animal, without the confounding affects of anesthesia.

Critical Parameters

If analyses are not performed on the day of blood collection, samples should be stored at −20°C (short-term, e.g., <2 months) or −80°C (long-term). Stability and storage depend on the different parameters to be determined. For example, free fatty acids are only stable for 2 to 3 days at −20°C.

Plasma samples can be used undiluted or diluted with deionized water if the volume is insufficient or the parameter to be measured exceeds accurate detection limits. If the blood is hemolyzed, a correction factor and/or resampling is suggested, as hemoglobin can interfere with certain measurements.

For plasma, a number of analyte measures favor the use of heparin over EDTA and vice versa, and this should be verified with each individual protocol before blood is drawn. For example, heparin can interfere with the assessment of plasma lipoprotein lipase activity, as heparin is a known inhibitor of this enzyme.

Because any invasive mouse technique should be considered potentially stressful, the same experimenter should perform the technique each time to minimize experimental variation due to animal handling skills. Since many factors such as centrifuge speed, clotting time, storage temperature, and time for blood processing may influence both serum and plasma preparations, carefully documented conditions of blood processing procedures should be followed to assure reproducibility of samples.

Anticipated Results

As tail vein sampling is less invasive than orbital eye bleed, multiple samplings should be well-tolerated by the mouse over the period of its lifetime. It is difficult to quantify how many samplings are possible or how much blood can be drawn at any one point in time. In general, however, the maximal volume of a single blood draw should not exceed 10% to 15% of total blood volume or 1% of the body weight of the animal. Restoration of normal blood volume takes 24 hr, but it may take up to 2 weeks for normal levels of erythrocytes and reticulocytes to be restored. Therefore, 2 weeks is the recommended minimum interval between consecutive maximal blood draws. Daily blood draws up to 1% of the total blood volume are acceptable, although the effects of stress, as well as the choice of collection site, need to be carefully considered. Blood loss exceeding 20% to 25% of total volume results in hypovolemia and cardiac failure due to decreased blood pressure, cardiac output, and oxygen delivery to vital organs.

The ratio of blood cells to plasma is typically 1:1, but a researcher should safely estimate that only ~50 µl of plasma will be obtained from 150 µl blood. Note that quality should not be sacrificed for quantity by removing too much plasma near the buffy coat and red blood cell layers and risking contamination. For automated analyzers such as for lipid analysis, this amount of plasma/serum is sufficient to perform a basic biochemical and lipid profile. Although the low yield of plasma from tail vein sampling is perceived as a drawback when multiple analytes are to be measured, the technique is ideal when multiple samples are required over a short period of time and the potential confounding effects of anesthesia are undesired. For example, the tail vein technique is ideal for a glucose tolerance test, which requires as many as eight blood samplings over 180 minutes but requires only 5 µl of blood per time point.

Time Considerations

For blood draw volumes and frequencies, see Anticipated Results. Because the same experimenter should perform all blood draws, the maximum number of animals that can undergo blood draw at any time will depend on the skills of the experimenter. An experienced blood drawer might be able to obtain samples from as many as 20 to 30 animals in 1 hr. The whole experiment should be performed within a reasonable time frame to avoid any confounding effects of changes in the animals due to diurnal rhythm and length of fasting.

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METABOLIC EXPLORATION OF THE MOUSE

Endocrine and metabolic dysfunctions are among the most common diseases in developed societies. Examples of such diseases include obesity, insulin resistance, type 2 diabetes, hyperlipidemia, and atherosclerosis. Mouse models are increasingly popular tools for studying and characterizing the molecular and physiological aspects of these diseases. The characterization of endocrine and metabolic dysfunction in mice has become of utmost importance for both basic and clinical scientists. This section will describe phenotyping protocols for the evaluation of numerous metabolic parameters including energy homeostasis, serum and tissue lipids, glucose homeostasis, and select pathophysiology. Many of these protocols are based on clinical tests used to evaluate human patients with metabolic diseases.

Despite the ease of mouse manipulation, the investigator should take heed that endocrine and metabolic disturbances are often subtle in their presentation, and are thus a challenge to detect even for the best mouse physiologists. In humans, clinical history and physical examination constitute critical elements in the general diagnostic process. Although a clinical history in mice is impossible, physical examination, as with humans, can provide important hints about endocrine or metabolic dysfunction. Numerous examples illustrate this principle nicely. For example, diseases of hormone deficiency or excess are physiologic determinants of physical traits including stature, weight, complexion, hairiness, and behavior. Body aspects such as thinness or obesity can result from or be associated with abnormalities in lipid or glucose metabolism. Critical observation of mouse behavior also reveals important information. Therefore, initial metabolic exploration should begin by an in-depth clinical analysis of the mouse in its home cage using tests such as SHIRPA or the dysmorphological screen (Hrabe de Angeles et al., 2000; Nolan et al., 2000). Screening of standard blood components like glucose, lipids, calcium, and electrolytes also gives clues for the diagnosis of endocrine or metabolic dysfunction, which can be further investigated with a wide array of more specialized and detailed tests covering the whole endocrine system and metabolism in general.

The acquisition of skills in endocrine and metabolic diagnosis is facilitated by familiarity with certain simple principles and rules. In particular, endocrine and metabolic disorders are most often seen as either an excess or deficiency of a certain biologically active hormone or metabolite. Alternatively, these disorders can be the consequence of inadequate cellular responses to a particular hormone or metabolite. These principles help guide the phenotyping paradigms summarized in the following units.

Hormones and metabolites can be characterized by the pathway and location of their production, their storage and release into the serum, and their degradation and metabolism. Although hormone and metabolite levels can be regulated in all of these aspects, production rate is often the primordial factor determining their levels. It is interesting that production of most metabolites and hormones is regulated directly or indirectly by the metabolite or hormone in question through interconnected positive and negative feedback loops. This control has important implications, since plasma levels of a metabolite or hormone only make sense if the appropriate regulatory factor is taken into account (e.g., glucose and insulin). Elevation of both metabolites/hormones and their regulatory factors is often reflective of a resistant state (e.g., glucose and insulin elevation in insulin

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resistance). A final interesting correlate of regulated hormone secretion is that it is the basis for various dynamic endocrine or metabolic tests for the evaluation of discrete disturbances in metabolic and endocrine function (e.g., intraperitoneal insulin tolerance test).

UNIT 29B.1

Evaluation of Energy Homeostasis

The critical factors contributing to body-weight maintenance include caloric intake and energy expenditure (Spiegelman and Flier, 2001). Alteration of either of these factors can upset the balance of the equation and result in body weight changes. Thus, the evaluation of body weight gain/loss over a period of time can be the most obvious indication of an alteration in energy homeostasis, and is typically coupled with other direct measurements such as food intake, body mass index, and fecal lipid content (see Basic Protocol 1). More sophisticated, noninvasive measurements of body composition that break down the weight in terms of body fat, lean mass, and bone mass can be obtained using dual-energy X-ray absorptiometry (DEXA; see Basic Protocol 3) or quantitative nuclear magnetic resonance (QNMR; see Alternate Protocol). To investigate the causes of energy dysregulation, several tests can be employed. Food-intake measurements such as those described in Basic Protocol 1 can indicate whether changes in caloric intake are contributing to the altered energy balance, whereas indirect calorimetry (see Basic Protocol 4) and the cold test (see Basic Protocol 2) assess the animal's expenditure of energy under basal and challenged conditions, respectively.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

STRATEGIC PLANNING

In vivo mouse experiments require a significant amount of strategic planning. To begin with, all in vivo experiments must be done in accordance with the appropriate animal care and use guidelines as defined by the country and institution where the work is carried out, and the time to acquire these approvals must be taken into consideration. Moreover, several parameters must be considered when designing a mouse experiment, which depend on the nature of the experiment. A minimum list of parameters to consider when planning the generation of a cohort of experimental animals for metabolic exploration includes mouse strain, gender, age, control mice, diet, housing conditions, time of experiment, and number of mice. For more information, refer to Champy et al. (2004).

For any experimental cohort, several variables need to be carefully controlled, such as the number of mice and their age, sex, and diet (Champy et al., 2004). In general, for metabolic exploration, male or female animals are tested after sexual maturity (~6 to 8 weeks), under chow, high-fat, and/or high-sucrose diet conditions. Preferably, all genotypes of animals should be derived from age-matched littermates with appropriate cohort sizes, since it is clear that there is substantial inter-animal variability. According to the authors' own experience and previously published work with C57BL/6J mice and power calculations, a group size of approximately ten to twelve animals is generally sufficient to observe statistically significant differences of the distinct interventions on metabolic and molecular parameters (Koutnikova et al., 2003; Cock et al., 2004). To be cautious and account for animals that might die in the course of these often long-term experiments, twelve animals per group are suggested; however, each individual user must determine the optimal number. In order to generate such a colony, depending on the size of litters and fertility of the animals, at least six to eight females will need to be bred at any one time. In cases where the number of animals is limiting, a consecutive

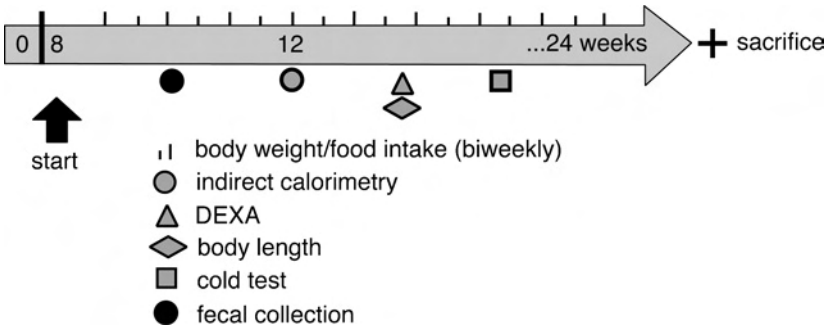

Figure 29B.1.1 A hypothetical timeline for a phenotypic analysis on a cohort of animals.

experimental strategy can be employed whereby animals are first tested under chow-fed conditions before putting them on the dietary challenge. Finally, a timeline outlining the experimental manipulations must be designed that minimizes the stress level of the animal. An example of such a timeline is provided for the procedures described in this unit (Fig. 29B.1.1); additional tests not discussed in this section can also be incorporated.

MEASUREMENT OF BODY WEIGHT, FOOD INTAKE, BODY MASS INDEX, AND FECAL LIPID CONTENT

BASIC PROTOCOL 1

Monitoring of body weight and food intake involves relatively simple tests that provide the first indications about metabolic homeostasis. The authors recommend initially evaluating body weight and food intake during a 6-week period, between 6 and 12 weeks of age. Body weight should be recorded twice a week at the same time of day. Care should be taken to avoid stressing the animals, since this can impact body weight gain. Twice-weekly weighing of the food given and the food remaining in the grid of the cages is a reliable method for evaluating food consumption. In general, food intake is measured when mice are housed at a density of four per cage. In the case of longer studies (e.g., aging), the frequency of weighing can be reduced to bimonthly recordings, and food intake can be measured less often, depending on results of initial testing.

Fecal lipid content is measured gravimetrically following a Folch extraction (Folch et al., 1951; Ishibashi et al., 1996). The authors recommend that feces be collected in parallel on a day when food intake is recorded, in order to measure lipid absorption. As an alternative to measuring total lipid gravimetrically, the fat extract can be partitioned according to the type of lipid (i.e., triglyceride and cholesterol) by subjecting it to enzymatic assays, thin-layer chromatography, or gas chromatography. These methods are beyond the discussion of this unit, although enzymatic assays will be included in a later unit on glucose and lipid homeostasis. A very sensitive measure of cholesterol absorption involves oral gavages of a mixture of dual-labeled [^{3}H]sitostanol and [^{14}C]cholesterol followed by determination of the stool sterol ratio of ^{14}C to ^{3}H (Schwarz et al., 2001). Whole-animal sterol synthesis rates can also be measured in vivo by determining the rate of incorporation of $^{3}H_2O$ into sterols over time (see Schwarz et al., 2001, and references therein). Finally, alternatives to the Folch lipid extraction protocol can be employed. For example, increased extraction of total fat and phospholipid can be achieved by sequential extraction with solvents chosen to optimize extraction of medium- and long-chain triglycerides and fatty acids and solvents designed to recover phospholipids (Chen and Innis, 2004).

CAUTION: Appropriate procedures for the use of radioactivity must be followed (see *APPENDIX 1F*).

Materials

Mice
Mouse food
0.1 mCi/ml [carboxyl-^{14}C]triolein (112 mCi/mmol)
2:1 (v/v) chloroform/methanol

Container to accommodate mouse during weighing
Scale accurate to 0.01 g
Mouse cages (e.g., 24 × 38 × 20–cm) with metal floor grids (grids or screens can be custom cut to fit bottom of cage; optimal grid size is one that allows feces to pass through but is still comfortable for mouse to rest on)
Inhalation anesthesia unit (composed of a mixing and flow-controlled system for O_2/isoflurane anesthesia; e.g., TEM, *http://www.TEM.fr*)
Digital caliper (range, 0 to 20 cm; accuracy, <0.02 mm; resolution, <0.01 mm; Ted Pella, Inc.)
Analytical balance accurate to 0.0001 g
70°C vacuum oven
10-ml Erlenmeyer flasks
60°C shaking water bath
Whatman no. 1 filter paper
10-ml glass test tubes with ground glass stoppers
Clinical tabletop centrifuge
Long-stem glass Pasteur pipets
Nitrogen evaporator (e.g., N-EVAP; Organomation Associates) with dry bath at 50°C
Preweighed glass scintillation vials
β-counter and scintillation cocktail

Measure body weight

1. Place a mouse container on a scale with an accuracy of 0.01 g and calibrate to zero.
2. Place the mouse in the container and record the weight (to the same accuracy) after the measurement stabilizes.

Measure food intake

3. Place the mice in a clean standard cage (e.g., 24 × 38 × 20–cm) with a metal floor grid, optimally at four mice per cage, with free access to water.
4. Weigh a portion of food appropriate for the feeding interval (e.g., 24, 48, or 72 hr) to an accuracy of 0.001 g, and place it in the grid of the cage. Assume an average mouse consumes 3 to 5 g/day. Place a lid over the grid to prevent the food from spilling over.

   For long-term food intake studies, the food quantity should be checked daily and supplementations weighed.

5. At the end of the feeding interval, remove the grid and weigh the remaining food to an accuracy of 0.001 g.
6. Calculate the amount of food consumed per mouse per day by dividing food consumed (weight of food given – weight of food remaining) by the number of animals per cage and by the number of days in the feeding interval.
7. Calculate kilocalories (kcal) of food consumed by multiplying the amount of food consumed by the caloric density of the food as provided by the manufacturer.

   For example, for a chow diet of 3.37 kcal/g with 5 g food consumed, the number of kcal consumed is 16.85 kcal.

As an alternative, systems designed to specifically record and analyze liquid and food consumption can be used—e.g., Columbus Instruments Lick Counter and Feed Monitor (http://www.colinst.com/) and TSE Systems Drinking and Feeding Monitoring System (http://www.tse-systems.com/). These systems generally allow for an unlimited number of measurements to be monitored by a computer.

Measure body mass index (BMI)

8. Determine the weight of each mouse as described in steps 1 and 2.
9. Place the mouse in an inhalation anesthesia unit and anesthetize using 3.5% to 4.5% (v/v) isoflurane in O_2 for induction and 1.5% to 3.0% (v/v) isoflurane in O_2 for maintenance (Flecknell, 1993).
10. Remove the mouse from the anesthesia unit and place it in a ventral position. Gently push the back of the mouse flat and straighten the spine. Using a digital caliper, determine the nasal-to-anal length of the mouse in cm.
11. Calculate the body mass index (in g/cm^2) by dividing the body weight by the square of the nasal-to-anal length.

Measure fecal lipid content

12. Place the mice in clean cages containing a metal floor grid instead of bedding.
13. Collect feces over a 24-hr period in parallel with a food intake measurement (see above) in order to determine fat balance (lipid intake and output). Dry the collected feces for 1 hr in a vacuum oven at 70°C, then clean the feces to remove remains of cage bedding and/or food pieces. Weigh total fecal output to an accuracy of 0.0001 g using an analytical balance.

    Preferably, fat balance should be determined on several consecutive days to account for the variability in intestinal motility.

14. Carefully weigh a 100-mg aliquot of droppings into a 10-ml Erlenmeyer flask, and mix with 5 µl of 0.1 mCi/ml [carboxyl-^{14}C]triolein (radioactive tracer).

    To ensure that fecal aliquots are representative of the whole group, duplicate or triplicate samples should be tested.

15. Cool the sample and extract by adding 2 ml of 2:1 chloroform/methanol. Incubate 30 min at 60°C with constant agitation in a shaking water bath.
16. Pass the sample by gravity through a Whatman no. 1 filter paper into a 10-ml glass test tube. Add 2:1 chloroform/methanol up to a final volume of 4 ml.
17. Back-extract the sample by adding 1 ml water and mix well by vortexing. Induce phase separation by centrifuging 10 min at 2000 rpm in a clinical tabletop centrifuge, room temperature.

    When equilibrium has been reached, there will be an upper transparent water/methanol phase, a lower chloroform phase, and an accumulation of material ("fluff") at the interface.

18. Carefully draw off the bottom organic phase using a long-stem glass Pasteur pipet with its tip placed at the bottom of the tube, and transfer to a new tube. Evaporate the sample to dryness using a nitrogen evaporator (e.g., N-EVAP) with dry bath at 50°C. Resuspend the residue in 2 ml of 2:1 chloroform/methanol.
19. Transfer the solution into a preweighed glass scintillation vial and evaporate the solvent using a nitrogen evaporator (e.g., N-EVAP) with dry bath at 50°C. Dry the vial in a vacuum oven at 70°C for 1 hr, then carefully reweigh the vial.

20. Determine the difference in weight between the empty vial and the vial containing the dried lipid to give the amount of fecal lipid in mg.
21. To correct for loss of lipids during extraction, determine the percent recovery of radiolabeled triolein. Add scintillation cocktail to the vial and count the radioactivity in a β-counter. Adjust the weight of the fecal lipid in each sample according to the percent of triolein recovery.
22. Using the corrected amount of fecal lipid, express fecal lipid content as a percent of the weight of the starting fecal aliquot (100 mg).
23. Determine the total amount of fat excreted in the 24-hr period by multiplying the percent fecal lipid by the total fecal output (step 2).
24. Subtract the total fat excreted from the total amount of fat consumed in 24 hr (based on the fat content of the food as provided by the manufacturer) to give the amount of retained fat. Express as a percent of fat consumed.

BASIC PROTOCOL 2

ASSESSMENT OF THERMOREGULATION BY THE COLD TEST

The cold test allows the evaluation of adaptive thermoregulation (Picard et al., 2002). In practice, mice are placed in individual cages without food but with free access to water. Initial body temperature is recorded by inserting a small thermoprobe into the rectum of the animal. Mice are then placed in a cold room at 4°C and rectal body temperature is recorded every hour during a 6-hr period. Results are presented as the curve of body temperature decrease over time. The authors do not normalize the cold test to body weight, but interpretation of the data should take into account whether there are differences in body weight.

Materials

Mice
70% (v/v) isopropanol
Mouse food
Container to accommodate mouse during weighing
Scale accurate to 0.1 g
Standard mouse cages (metal grid not required)
Thermometer with thermoprobe (accuracy <0.1°C) and rectal adapter (e.g., Bioseb; *http://www.bioseb.com*)

1. Determine the weight of the mouse as described in Basic Protocol 1, steps 1 and 2.
2. Place each test mouse individually in a clean cage, without food but with free access to water.
3. Disinfect the thermoprobe by washing it with a 70% isopropanol solution.
4. Measure the initial body temperature (T_0) of each mouse by inserting the thermoprobe into the rectum and recording the temperature on the probe once the value has stabilized (3 to 4 sec).
5. Place the individually caged mice in a cold room at 4° ± 1°C.
6. Measure and record the rectal temperature hourly, while in the cold room, over 6 hr.

   The six measurements thus obtained are designated T_1, T_2, T_3, T_4, T_5, and T_6.

7. At the end of the experiment, place the mice into new cages in a room with normal ambient temperature (they can be regrouped if required), provide with food and water, and ensure that the animals exhibit normal behavior.

   If the body temperature becomes too low (<25°C), place the cages close to a 37°C heating source for 15 to 30 min to warm the animals.

DETERMINATION OF BODY COMPOSITION BY DUAL-ENERGY X-RAY ABSORPTIOMETRY

BASIC PROTOCOL 3

Bone densitometry and body composition (lean and fat content) are often measured using dual-energy X-ray absorptiometry (DEXA) analysis (Grier et al., 1996; Nagy and Clair, 2000). DEXA analysis can be performed using ultra-high-resolution densitometers such as those made by GE Medical Systems (e.g., PIXImus; *http://www.gehealthcare.com/inen/rad/bonedens/peripheral/piximus.html*) or Norland Medical Systems (pDEXA Sabre X-ray, *http://www.norland.com*) that allow for precise measurements of bone mineral and body composition from body imaging in less than 5 min in anesthetized mice.

CAUTION: Specific X-ray safety procedures should be followed.

Materials

Anesthetic solution (see recipe)
Mice
PIXImus densitometer (0.18 × 0.18 mm; GE Medical Systems)
Computer with Windows PIXImus software (e.g., version 1.4x; GE Medical Systems)
Container to accommodate mouse during weighing
Scale accurate to 0.1 g
1-ml syringe with 25-G, 0.5-mm needle
Specimen tray for PIXImus (GE Medical Systems)

Prepare the PIXImus analyzer

1. Turn on the PIXImus analyzer at least 2 hr prior to testing.
2. Initiate the software by double-clicking the PIXImus icon.
3. Calibrate the PIXImus analyzer and perform quality control according to the operator's manual.

   The field calibration procedure takes ~60 min. Each PIXimus has a "phantom," i.e., a structure with predetermined bone mineral density and fat content, which is analyzed and used for quality-control purposes.

Anesthetize the mouse

4. Determine the weight of the mouse as described in Basic Protocol 1, steps 1 and 2. Calculate and record the volume of anesthetic solution required for injection at 8 ml of anesthetic solution per kg.

   When prepared as described and administered at 8 ml/kg, animals receive a dose of 200 mg/kg ketamine and 10 mg/kg xylazine.

5. Administer the appropriate volume of anesthetic solution by intraperitoneal injection using a 1-ml syringe and 25-G, 0.5-mm needle.

Perform the measurements

6. Remove the adhesive backing from the specimen tray, place the anesthetized mouse in a ventral position on the tray, and ensure that the vertebral column is straight. Wrap the mouse's tail around itself and extend the legs away from its body.

7. Position the tray in the scanner so that the head is oriented towards the left, from the investigator's point of view.

8. Start the measurement according to the PIXImus operator's manual.

   The image acquisition takes less than 5 min.

Perform the calculations and allow animals to recover

9. Following image acquisition, determine the bone mineral density (BMD, in g/cm^2), bone mineral content (BMC, in mg), bone area (in cm^2), and body composition (% fat and lean, and fat tissue in g) with the PIXImus software.

   The specific body imaging area or the region of interest (ROI) can be manually selected within the total body image. For example, to image the spine and femur, the software positions an oval-shaped exclusion area to exclude the head and its soft tissue.

10. At the end of the experiment, remove the mouse from the plastic tray and return it to its home cage. Ensure that the test mice recover from the anesthetic and exhibit normal behavior.

    Generally mice are allowed to recover on a 37°C heated surface.

ALTERNATE PROTOCOL

DETERMINATION OF BODY COMPOSITION BY QUANTITATIVE NUCLEAR MAGNETIC RESONANCE

As an alternative to DEXA (Basic Protocol 3), new EchoMRI quantitative magnetic resonance (QNMR) methods (e.g., Bruker Minispec NMR analyzer) can also be employed for the in vivo analysis of whole-body composition (lean and fat content) of conscious live mice. Both the DEXA and the EchoMRI methods eliminate the need for destructive chemical analysis and allow for multiple consecutive measurements throughout the lifetime of the animal.

Materials

Mice
Control/calibration sample (Bruker)
Bruker Minispec NMR analyzer (*http://www.minispec.com/mq/mice.html*), interfaced with personal computer running MS Windows 2000 or higher with Minispec software package installed

1. Turn on the NMR and then the computer.

   Initially, three lights appear on the NMR indicating the preheating stage (red), the transition to normal heating (yellow), or the correct temperature (green). The NMR is ready to use ~3 hr after powering up.

2. Click the Minispec icon on the computer to load the Minispec software, then click the Daily Check icon to calibrate the machine.

3. Confirm that the control tube has been inserted into the NMR and click OK when prompted by the "Confirm minispec Daily Check sample has been inserted" message.

4. When calibration is finished, click Yes to print the results when prompted by the "minispec Daily Check OK: print out results" message.

   When the calibration is not successful, an error message appears and action should be taken accordingly. Refer to troubleshooting section in the Minispec mcSeries User's Manual.

5. Weigh the mouse as described in Basic Protocol 1, steps 1 and 2. Remove the Control sample tube and insert the weighed animal into the Sample tube. Slowly insert the plunger, being careful not to drop the plunger onto the animal.

6. Insert the Sample tube, then click OK when prompted by the message "Waiting for inserting sample."
7. Enter the animal's identification number and the operator's initials when the messages "Input sample ID" and "Input operator sample" appear. Click on Measure to initiate testing.

   Testing takes ~3 min per animal.

8. When all testing is finished, remove the Sample tube, return the mouse to its home cage, and verify that it behaves normally. Repeat steps 5 to 8 until all animals have been tested.
9. Select the Save As option under the File menu, give the data a file name, check the "data format" radio button, and click Save. Shut down the Minispec program by selecting Exit from the File menu.

INDIRECT CALORIMETRY BY OXYMAX

BASIC PROTOCOL 4

Energy expenditure is evaluated through indirect calorimetry by measuring oxygen consumption with a specific apparatus such as the Oxymax (Columbus Instruments; *http://www.respirometer.com/microoxy.html*) or CaloSys (TSE Systems; *http://www.Tse-systems.com/calorimetry/calorimetry.htm*; Porter, 2001). These systems monitor oxygen (O_2) and carbon dioxide (CO_2) gas volume concentrations at the inlet and outlet ports of a partially sealed chamber through which a known flow of ambient air is forcibly ventilated, either by positive pressure or by drawing the flow from the chamber. The concentration differences measured between the ports, along with the flow information, are used to compute oxygen consumption (VO_2) and carbon dioxide production (VCO_2), and the respiratory quotient (RQ) is calculated as VO_2/VCO_2. Chambers are equipped with a third port for temperature measurement.

In practice, mice are placed individually in the metabolic chambers while they have ad libitum access to food and water. Ideally, indirect calorimetry should be performed at thermoneutrality (~30°). Thermoneutrality is defined as the range of ambient temperature at which energy expenditure is lowest, no metabolic heat is required to maintain body temperature, and there is minimal oxygen consumption. At this temperature, the animal can maintain homeostasis without excessive use of energy for thermoregulation, and, hence, energy is available for maintaining optimum conditions of health and performance. When the heat load changes, the animal must expend energy to either eliminate excess heat or make heat in order to maintain internal thermal balance. Therefore, body temperature changes can affect various body functions (Gordon, 1993). If the temperature drops below 30°C, mice show an increase in metabolic rate, which can confound the outcome of the tests. The actual temperature for thermoneutrality can vary in rodents anywhere from 28° to 32°C. In general, higher temperatures should not be used. If indirect calorimetry cannot be performed at thermoneutrality, ambient temperature of (23° to 24°C) should be used. Indirect calorimetry is preferably performed for a 24-hr period while the mice have free access to food. O_2 and CO_2 measurements are taken at regular intervals during the whole experiment, and VO_2 and VCO_2 values are expressed as ml/kg/hr. As energy expenditure varies with body weight, it is important to normalize the values obtained for body mass.

The measurement of metabolic performance by calorimetry can be performed simultaneously with measurement of home cage activity and drinking and feeding behavior using modified systems—e.g., LabMaster (TSE Systems; *http://www.tse-systems.com/labmaster/labmaster.htm*) and Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments; *http://www.colinst.com/brief.php?id=61*). In

these types of systems, measurements of O_2 and CO_2 concentration, air flow, and temperature are recorded along with total activity (ambulatory and fine movements and rearing), food and liquid consumption, and urine and feces excretion. These systems, when available, can provide a large amount of data in a short period of time.

Materials

Mice
Container to accommodate mouse during weighing
Scale (capable of weighing 1 to 50 g to an accuracy of 0.1 g)
Respirometer: Oxymax system (Columbus Instruments; *http://www.respirometer.com/microoxy.html*)
Computer with the Windows Oxymax software
Calibration gases: one tank with 20.5% O_2/0.8% CO_2 and a second tank with 100% N_2

1. Determine the weight of each mouse as described in Basic Protocol 1, steps 1 and 2.

   As energy expenditure varies with body weight, values are normalized to body mass (see step 7).

2. In an experimentation room set to 30°C, place each mouse individually in an airtight cage (supplied with the Oxymax respirometer) with free access to food and water. Seal the cages tightly, taking care to ensure minimum leakage of gases.

3. Warm up the Oxymax respirometer for ≥2 hr, then calibrate the CO_2 and O_2 sensors according to the Oxymax operating manual prior to the start of the experiment.

   The calibration of the gas sensors and flow meters is required to ensure precise measurements and involves using a gas mixture of 20.5% O_2 and 0.8% CO_2 (balance N_2) to adjust the O_2 and CO_2 sensors to reflect the contents of the calibration gas.

4. Following calibration, click the Experiment Configuration tab. Select the Chamber Configuration tab, and click on Chamber One. Enter the mouse information, including ID, weight, and sex, and then click Apply. Continue this procedure until each chamber is entered, then save the data file.

5. Ensure that the chambers are connected according to the Oxymax operating manual, and start the system by clicking Run.

   Measurements of O_2 and CO_2, in units of ml/kg/hr, are acquired at regular intervals. The time interval is selected by the user, but is generally 3 min. The total duration of the recording is ∼24 hr, which covers both active and resting phases.

6. At the end of the experiment, click Stop, shut off the air, and remove the mice from the chambers. Return to home cages and ensure that they exhibit normal behavior.

   Chambers should be carefully washed and disinfected between experiments.

7. Normalize the values of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) to body mass (ml/kg$^{0.75}$/hr or ml/kg$^{0.67}$/hr; see Critical Parameters).

   Normalization is done automatically by the instrument software. In some cases (obese animals) values can be normalized to lean body mass as measured by DEXA analysis.

   The respiratory exchange ratio (RER), also known as the respiratory quotient (RQ), is calculated as VO_2/VCO_2. Heat is calculated from the VO_2 and RER information using the standard formula: Heat (H) = calorific value (CV) × VO_2 × 0.001, where CV = 3.815 + 1.232 × RER. RER and heat are calculated automatically by the instrument software for each time interval. In this way, the differences in resting and active periods can be seen.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Anesthetic solution

1.4 ml ketamine
0.35 ml 2% (w/v) xylazine
3.85 ml 0.9% (w/v) NaCl (sterile)
Prepare fresh

COMMENTARY

Background Information

The evolution of body weight gain is a critical first-line phenotyping test. Body mass and composition reflect the combined effects of three processes: energy intake, energy partitioning (storage), and energy expenditure. Body-weight evolution, to a large extent reflective of fat stores, represents the net balance between energy intake and energy expenditure. When a loss or gain of weight is evident, or when body composition is altered, a mismatch between energy intake and expenditure should be excluded. The phenotyping protocols outlined here provide a starting point for evaluating all sides of the equation and can provide a direction for subsequent molecular analysis.

Energy intake. The energy required for maintaining body function both at rest and during stress and exercise is provided in the form of carbohydrates, fat, and protein nutrients that are consumed daily (Spiegelman and Flier, 2001). In general, energy intake and energy expenditure are closely matched, such that body weight is remarkably stable even in the face of large fluctuations in caloric intake. The tight matching of energy intake and energy expenditure is governed by the central nervous system, primarily in the hypothalamus (Coppari et al., 2005). Leptin and insulin are primary effectors that activate receptors located on secondary effector neurons in the hypothalamus; these neurons express neuropeptides with long-term effects on food intake and body weight. Orexogenic neuropeptides that increase food intake, such as neuropeptide Y (NPY), are inhibited by leptin and insulin, while aneroxogenic neuropeptides, such as proopiomelanocortin (POMC), are up-regulated. These neuropeptides subsequently act on local receptor populations to exert their physiological effects. As circulating insulin and leptin levels are proportional to the amount of body fat, they are critical sensors of long-term energy stores (adipose tissue). In parallel with these more long-term regulatory signals emanating from the body's energy stores, a number of short-lived satiety factors originate within the gastrointestinal tract. For example, cholecystokinin, glucagon-like peptide, and ghrelin modulate short-term food intake by signaling to the CNS through vagal or sympathetic afferents, as well as through the circulation. Determining changes in food intake and food (lipid) absorption by measuring lipids excreted in the feces is the starting point for the evaluation of energy homeostasis.

Energy storage. The main organ used to store energy ingested in excess of immediate need is adipose tissue (Havel, 2002). In general, the energy density of adipose tissue (containing mainly fat) is >10-fold higher than that of muscle (which is principally composed of protein) or the liver (containing glycogen). The hypothalamic centers also monitor energy stores. For example, serum leptin increases with adipose tissue mass, thus informing the brain of the amount of energy stored. The role of leptin in signaling the brain about chronic changes in energy status is complemented by that of insulin, which conveys additional information about long-term changes in peripheral metabolism to the brain. The subsequent manipulation of energy stores is governed by a wide variety of enzymes, transcription factors, cytokines, and receptors. For example, changes in PPARγ activity can alter adipocyte differentiation and, ultimately, fat stores. Techniques such as DEXA and QNMR are phenotyping tests that distinguish body composition according to lean and fat, and which can thus identify defects in the distribution or partitioning of stored/chemical energy when energy balance is challenged.

Energy expenditure. The third part of the energy balance equation is energy expenditure (Lowell and Spiegelman, 2000). Energy expenditure consists of several components, roughly separated according to obligatory basal activity (for performance of cellular

and organ functions), physical activity, and adaptive thermogenesis. Whereas energy enters the organism as food, it exits as heat and/or work on the environment. Fuel is converted step-by-step into ATP, which is then used for biological work within the cell; one side product of this reaction is heat. Energy expenditure at rest changes markedly in response to environmental temperature. Part of the response is due to shivering, but another part results from increased adaptive thermogenesis. Adaptive thermogenesis is defined as heat production in response to environmental temperature or diet. It serves to protect the organism from cold exposure or to regulate energy balance after changes in diet. In rodents, brown adipose tissue is a major site of adaptive thermogenesis. A real-life example of an extended cold test such as that described in Basic Protocol 2 is provided by animals that hibernate, such as the bear. Hibernating animals maintain a steady body temperature even though the temperature of the environment around them changes constantly. This production of heat is called non-shivering thermogenesis. The brain detects alterations in environmental temperature and diet, and, through neural circuits, activates efferent pathways to control energy expenditure. The primary efferent pathway is believed to be the sympathetic nervous system, in addition to the hypothalamic-pituitary-thyroid axis. Regulation of energy expenditure is in part achieved by tightly coupling the reactions in energy metabolism. For example, for any given molecule of fuel, a fixed amount of NADH and FADH is generated, which translates into a fixed number of protons that are pumped across the mitochondrial matrix by the electron transport chain. These protons re-enter via ATP synthase and produce a fixed number of ATP molecules that can be used to deliver a fixed amount of biological work. Because the complete combustion of food is achieved at the expense of molecular oxygen, the heat generated in these exergonic reactions can be estimated by measuring oxygen consumption using indirect calorimetry (see Basic Protocol 4). When this test is performed at rest and at thermoneutrality, it reflects mainly obligatory basal energy expenditure and modest physical activity. The cold test (see Basic Protocol 2) is one measure of adaptive thermogenesis and can provide stress that may reveal a change in energy homeostasis not seen under the experimental conditions of calorimetry. When energy expenditure is altered, as indicated by calorimetry or cold tests, there is either an "uncoupling" of one of the reactions of cellular metabolism or a change in the amount of biological work. For example, uncoupling protein 1 (UCP1) has been shown to increase energy expenditure by uncoupling the relationship between protons entering the mitochondrial matrix and the synthesis of ATP (Lowell and Spiegelman, 2000).

Critical Parameters

Housing conditions and food intake. To determine the amount of food consumed per mouse, the total amount of food consumed per cage is divided by the number of animals per cage. Group housing can be criticized in view of issues of animal dominance as they affect food consumption. However, the authors have recently demonstrated that mice housed individually are severely stressed, which in turn significantly affects numerous parameters including blood insulin and glucose levels (Champy et al., 2004). In this instance, there is a "no win" situation regarding minimizing animal stress and maximizing the accuracy of the food intake measurement. The authors suggest that standard conditions always be used. For example, five mice cannot always be housed in one cage, especially when male littermates are being used, as males from different litters should not be housed together. Furthermore, unless the intention is to breed mice, females and males are never housed together. Thus, the number of cages and the number of mice per cage will likely change depending on the experimental cohort, and each investigator must make the best arrangement given the conditions (e.g., animal housing space, money, cohort size and distribution) available. Multiple tests can affect weight gain, so it is important to have weight values determined at times when minimal manipulations are being performed on the mouse cohorts. Comprehensive cage monitoring systems employing "metabolic cages" are an excellent alternative for automation of the measurement of food intake and various other metabolic parameters (including indirect calorimetry), thus minimizing overall animal stress and duration of the experiments. These metabolic cages can also be designed to study mouse movement and activity levels, as well as sleep patterns.

Body weight gain under calorie-dense diets. In order to accentuate differences in energy homeostasis, it is common practice to challenge mice with diets rich in caloric content. Such challenging conditions will

sometimes cause differences in body weight gain to become apparent. Unlike humans, mice in general do not like diets rich in fat and/or sucrose. This is because these diets often have a different consistency and taste from what they generally prefer. Mice usually like their food to be hard, as is the case with chow-like compositions; however, high-fat diets are generally soft. In the authors' experience, the total volume/mass of food consumed decreases when mice are switched from chow to a high-fat diet. However, despite the reduced mass of food consumed, total caloric intake increases due to the caloric density. In some instances, mice refuse to eat the new diet; to avoid this, the authors often fast mice overnight before switching to a different diet.

Body mass index. It is difficult to calculate the BMI because determining the length of a mouse is not simple. In many cases the differences will be very subtle, on the order of millimeters. In addition to measuring length by using a caliper, measurement can be obtained in parallel with DEXA and/or X-ray analysis, which require the mouse to be anaesthetized and imaged.

Fecal lipid content. The protocol described here is a rapid method for the determination of fat in feces. As the sensitivity of this assay depends on the ability to detect rather small differences in weight, care must be taken. In general, the percent recovery of radiolabeled triolein is 85% to 90%. If the recovery rate is not sufficient, an additional chloroform/methanol solvent extraction can be included and the organic phases combined.

Cold test. In general, this is a relatively simple test. Occasionally, when inserting the thermoprobe into the mouse, resistance is met due to the feces in the colon. In this instance, the lower belly of the mouse can be depressed somewhat to help with the fecal movement and excretion. As this is a fairly stressful test for mice, their health should be monitored throughout; if the body temperature of the mouse goes below 25°C, it should be removed from the cold and marked as "<25°C" for the remainder of the test. The number of mice used for the test depends on how quickly the measurements can be taken with the rectal probe and how long the investigator can stand the cold.

DEXA/QNMR. These tests can easily accommodate a large number of animals. In the case of DEXA, mice should be anesthetized in a staggered fashion, and care should be taken to ensure appropriate recovery. The actual data-acquisition steps for DEXA require <5 min. For QNMR, mice do not need to be anesthetized, and data acquisition is faster (<2 min). In view of this advantage and the slightly better accuracy, it is expected that QNMR will rapidly become the test of choice for determining body composition (Taicher et al., 2003).

Oxymax analysis. Based on allometric scaling methods, metabolic body mass was estimated as the body weight to the power of 0.75 or 0.67 (ml/kg$^{0.75}$/hr or ml/kg$^{0.67}$/hr; Porter, 2001). Ideally, however, VO_2 should be normalized to lean body mass and not total body mass. For groups of animals with roughly the same body composition and fat mass, normalization is not required. However, in obese animals, adipose tissue consists mainly of "metabolically inactive" storage tissue, and even normalization to body weight using the power of 0.75 or 0.67 gives an incorrect estimate of metabolic body mass. In such cases, lean body mass (which reflects "metabolically active tissue") is best obtained from the analysis of body composition as determined by densitometry or an equivalent method. Taking all this into account, the authors prefer to perform indirect calorimetry in young animals, at an age when body weight differences are usually less pronounced.

In order to account for the time the animal requires to habituate to the setting of the Oxymax and overcome stress, the first hour of measurement is generally excluded from the final analysis. In some instances, indirect calorimetry measures are performed over 72 hr; however, the stress associated with continued housing of the mouse alone and on metal floor grids must also be considered. Another consideration is the number of chambers in the indirect calorimetry system, which can vary from 8 to 60 chambers, thus greatly affecting the number of mice measured at one time.

Anticipated Results

A summary of all calculations pertaining to the specific phenotyping tests is presented in Table 29B.1.1.

Body weight and food and water intake. As mice age, they will gain weight (Fig. 29B.1.2). The question is whether the mutant or treated mice gain more or less as compared to the wild-type control mice. Data are generally presented as a growth curve showing the average weight of the mouse over time. In some cases, data are expressed as percent loss or gain in weight as compared to weight at the beginning of the experiment. The length of time for the experiment is generally 6 to 12 weeks. Since

Table 29B.1.1 A Summary of the Measures Used in the Evaluation of Energy Homeostasis

	Measure	Formula (units)
Body	Body weight	(in g)
	Length	(in cm)
	Body mass index (BMI)	body weight/length (in g/cm^2)
Food intake	Food consumed	[food given − food left over (in g)]/no. mice per cage
	Energy consumed	kcal/g of diet × g food consumed (in kcal)
Fat absorption	Fecal lipid content	Retained fat = total fat consumed − excreted fat (in g)
Body composition	Fat	(in g)
	Lean	(in g)
	% Fat	[Fat (in g)/total tissue mass (in g)] × 100
	Bone mineral density (BMD)	(in g/cm^2)
	Bone mineral content (BMC)	(in g)
	Area	(in cm^2)
Cold test	Body temperature	(in °C at T_1, T_2, T_3, T_4, T_5, T_6 expressed in hr)
Indirect calorimetry	VO_2	(in ml/kg$^{0.75}$/hr or ml/kg$^{0.67}$/hr)
	VCO_2	(in ml/kg$^{0.75}$/hr or ml/kg$^{0.67}$/hr)
	Respiratory exchange rate (RER)	VO_2/VCO_2

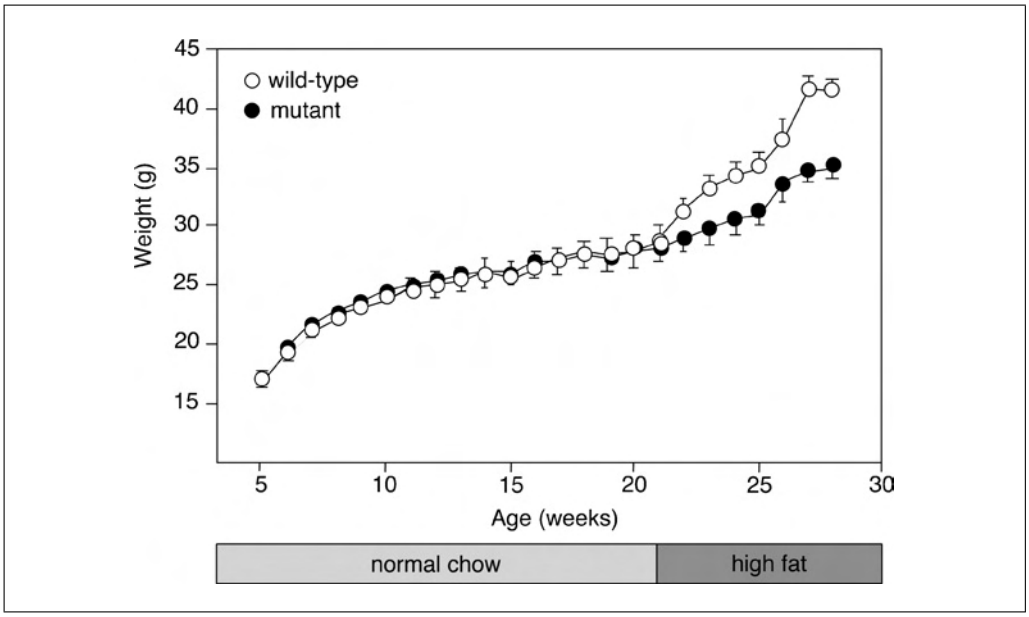

Figure 29B.1.2 An example of body-weight evolution in mice. A consecutive experimental strategy was employed whereby adult male wild-type and mutant mice were tested first under chow-fed conditions and then with a high-fat diet. Compared to wild-type mice, mutant mice are resistant to diet-induced obesity.

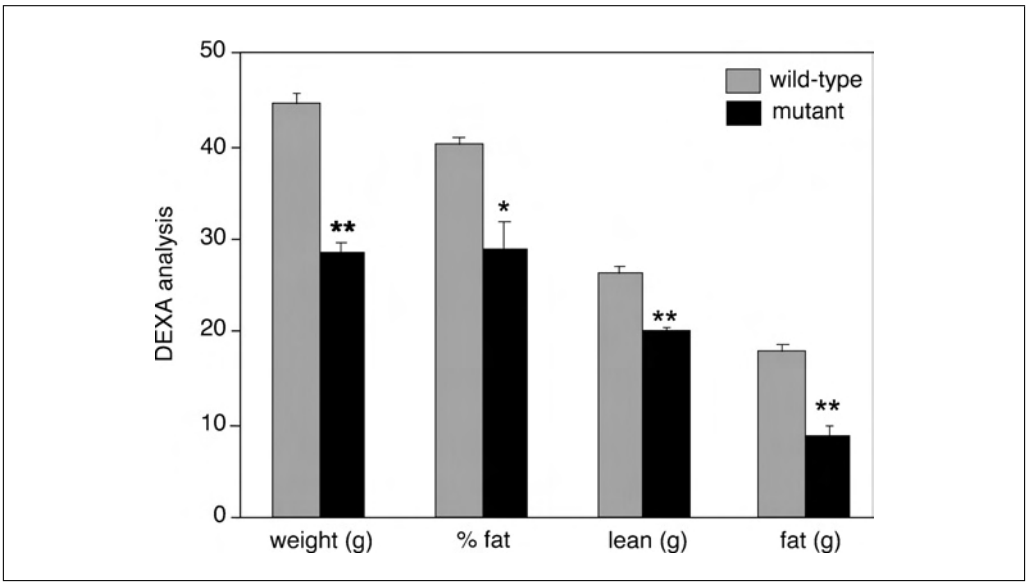

Figure 29B.1.3 An example of data obtained using DEXA analysis. Adult male wild-type and mutant mice were tested under high-fat diet conditions.

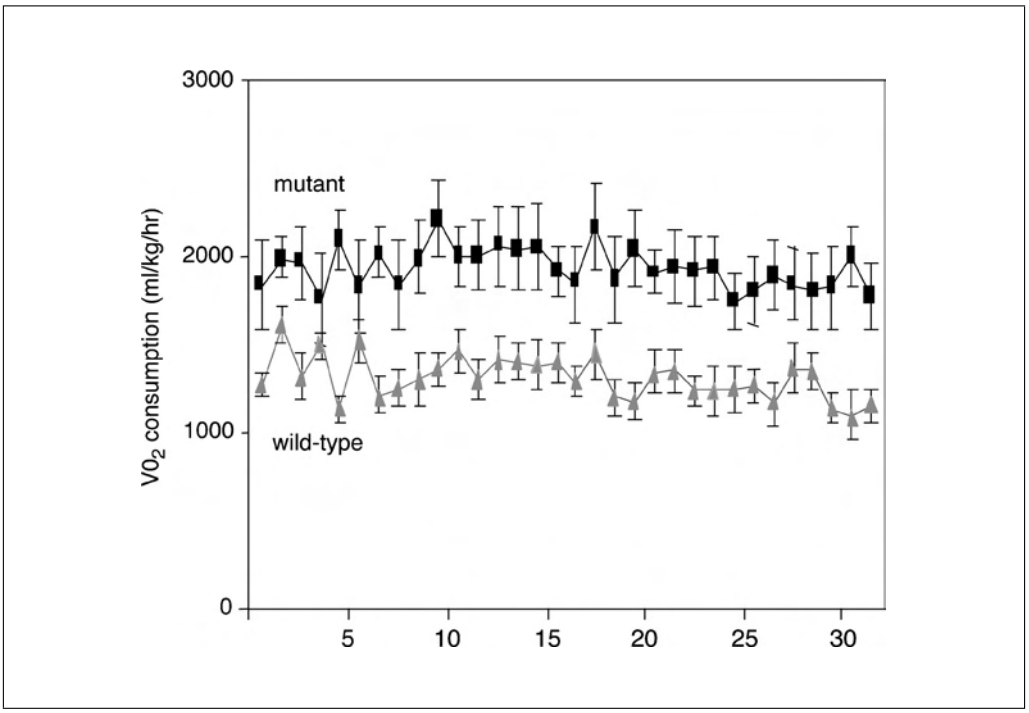

Figure 29B.1.4 An example of data obtained by Oxymax. Adult male wild-type and mutant mice were tested under high-fat diet conditions.

metabolic tests can be stressful and affect the weight, it is preferable to use weight data when the animals are undergoing a minimal number of manipulations. As stated above, calorie-rich diets can increase weight gain.

Fecal lipid content. Values of fecal lipid content in a mouse can vary greatly—anywhere from 20% to 50% of dry weight—and depend on numerous factors such as the type of diet, the genetic abnormality, and the age of the animal. When fecal lipid data are combined with food-intake experiments, altered patterns of food consumption versus absorption can be distinguished.

DEXA and QNMR analysis. When changes in body weight are detected, parallel changes in body composition should also be found (Fig. 29B.1.3).

Indirect calorimetry. The balance of energy must be accounted for. Therefore, when there are changes in body weight, changes should either be detected in caloric intake or

loss in feces, and/or in energy expenditure (Fig. 29B.1.4).

Time Considerations

Performing in vivo mouse experiments is not a trivial process. Considerable planning is required to acquire ethics approvals, not to mention the time needed to generate the experimental mouse cohorts. Compared with this, the time needed to perform the actual phenotyping experiments is usually limited, especially when the protocols have already been standardized and validated. However, it is important to design and adhere to a relevant timeline of experiments, such as that proposed in Figure 29B.1.1, in order to minimize animal stress. The use of a pilot feeding study whereby mouse weight is simply monitored over several weeks is useful in order to indicate whether subsequent full metabolic phenotyping is a priority in the experimental cohort under investigation.

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Lipid and Bile Acid Analysis

Clinical chemistry screening to identify changes in concentrations of serum lipids, including cholesterol, free fatty acids (FFA), and triglycerides (TG), is an important part of first-line metabolic phenotyping. Whereas cholesterol is an important body constituent necessary for membrane function and synthesis of hormones and bile acids, TG and FFA are important energy reservoirs. As a consequence of the pleiotropic functions of these lipids, many diseases have been associated with altered lipid levels. For example, elevated low-density lipoprotein (LDL) cholesterol levels increase the risk of atherosclerosis, a pathology characterized by lipid accumulation in the arterial wall (Grundy, 2004). When changes in serum lipids are observed, more detailed phenotyping screens, including lipoprotein profiling and tissue lipid content, can facilitate the identification of the underlying mechanism associated with the dyslipidemia, especially when combined with other phenotypic observations. Since bile acids are the most important pathway for eliminating cholesterol from the body, analysis of serum bile acid levels as well as bile acid pool size and composition is also valuable in a comprehensive lipid phenotyping screen. In addition, serum bile acid levels are an early and sensitive indicator for liver dysfunction. In this unit, methods associated with serum and tissue lipid analysis are outlined, as are protocols for assessing bile acids.

Basic Protocol 1 describes kit-based colorimetric procedures for determining TG, total cholesterol, LDL cholesterol, HDL cholesterol, and FFA. Alternate Protocol 1 describes similar lipid analyses using an automated laboratory workstation. Basic Protocol 2 describes separation of the different lipoprotein fractions by differential ultracentrifugation, while Alternate Protocol 2 describes a comparable fractionation procedure employing fast protein liquid chromatography (FPLC). Both of these procedures yield preparations that can be quantitated via Basic Protocol 1. Basic Protocol 3 describes extraction of lipids from tissue for analysis via Basic Protocol 1. Basic Protocol 4 and Alternate Protocol 3 describe two different methods for measurement of serum bile acids, while Alternate Protocol 4 provides a method for analyzing bile acid pool size and composition. Finally, a Support Protocol is provided for lipoprotein quantitation.

STRATEGIC PLANNING

Critical considerations with respect to the general planning of in vivo experiments and minimizing variation due to genotype and phenotype are discussed in *UNIT 29A.2*. Additional considerations in the context of this unit include those associated with blood drawing, such as the method and timing of blood collection and the length of fasting. As mice are small animals, their blood supply is correspondingly small. In general, the blood-draw technique chosen should give the required quantity of blood while minimizing invasiveness (see tail vein method in *UNIT 29A.3*). Although there is no hard and fast rule as to the maximum amount of blood that can be collected at any single point without endangering the mouse, anything over 10% to 15% of total blood volume or 1% of the body weight of the animal can induce hypovolemic shock and/or death. As blood lipid profiles reflect both diet-derived sources and de novo synthesis, a critical parameter to consider is the length of fasting prior to blood draw. For general analysis, a 4-hr fast at the end of the dark cycle is sufficient, as this allows for diet-derived lipids to be processed. A 4-hr fast will also limit responses to starvation, such as FFA release. Longer (overnight) and shorter fasts are also often used; however, data must be considered in the context of the concomitant metabolic consequences. Feeding, for instance, is associated with gallbladder contraction; therefore, fasting levels of plasma bile acids tend to be lower then those in the fed state. These considerations are critical anytime comparisons are to be made.

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BASIC PROTOCOL 1

MANUAL MEASUREMENT OF SERUM LIPIDS

Measurements of triglyceride (TG), total cholesterol, free fatty acids (FFA), and low- and high-density lipoprotein (LDL and HDL) cholesterol can be performed manually or with an automated laboratory workstation, such as the Olympus AU400 or the Cobas Integra (Roche Diagnostics), using specific colorimetric or enzymatic methods. In general, 80 to 150 μl of plasma is sufficient for all measurements, with the lowest sample quantities being required for the automated protocols. Many of the methods are provided as kits, such that all reagents, standards, controls, and protocols are provided by the manufacturer in a standardized and user-friendly format. Several different suppliers for these kits exist, e.g., Olympus, Cobas (Roche Diagnostics), Wako, and Sigma.

The approaches are generally based on similar principles, with differences mainly in the choice of chromophore and color of the spectra. For example, cholesterol can be measured using an enzymatic color test whereby cholesterol esters (CE) are hydrolyzed by cholesterol esterase to free cholesterol, which is then oxidized by cholesterol oxidase (CHO) to cholestene-3-one. This reaction simultaneously produces hydrogen peroxide, which then is coupled with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to yield a chromophore that is measured spectrophotometrically. TGs can be measured by a series of coupled enzymatic reactions beginning with hydrolysis by lipases to give glycerol and FFA. The glycerol is then phosphorylated by glycerol kinase to produce glycerol-3 phosphate, which is oxidized by molecular oxygen in the presence of glycerol phosphate oxidase (GPO) to produce hydrogen peroxide and dihydroxyacetone phosphate. The hydrogen peroxide is then used to oxidatively couple *p*-chlorophenol and 4-aminoantipyrin catalysis by POD to give a red dye that is measured spectrophotometrically. FFA are measured using an enzymatic color test whereby acyl CoA synthase is used to convert fatty acids into acyl CoA. In a second reaction, acyl CoA oxidase catalyzes the formation of 2,3-trans-enoyl CoA and hydrogen peroxide, the later product being subsequently reacted with 4-aminophenzone and MEHA and catalyzed by POD to give a red pigment whose intensity is proportional to the concentration of FFA in the sample. HDL cholesterol can be measured using an enzyme chromogen system whereby an anti-human β-lipoprotein antibody is used to bind all non-HDL lipoproteins or apoB-containing lipoproteins (chylomicrons, VLDL, IDL, and LDL). In the first reaction, the antigen-antibody complexes formed with the apoB-containing lipoproteins block all subsequent enzyme reactions, allowing HDL cholesterol to be specifically measured in a second reaction by the CHO/POD system. LDL cholesterol can be measured following a two-step reaction in which the first step uses cholesterol esterase and cholesterol oxidase to break down all non-LDL lipoproteins in the presence of an LDL-cholesterol protecting reagent. In the second step, the protecting reagent is released from LDL, and LDL cholesterol is quantified by the CHO/POD system.

Materials

Blood serum, plasma, or isolated lipid fraction obtained via other protocols in this unit

Lipid analysis kits (Wako):
- Total cholesterol kit
- LDL cholesterol kit
- HDL cholesterol kit
- FFA kit
- Free cholesterol kit
- TG kit

5-ml plastic or glass tubes

Spectrophotometer

Table 29B.2.1 Specifications for Lipid Measurements Using Wako Enzymatic Reaction Kits

Test	Sample/standard/ calibrator[a]	Reagent 1	Reagent 2	Incubation time/temperature	Absorbance (nm)
Total cholesterol	20 µl	2 ml	0	5 min, 37°C	600
Free cholesterol	50 µl	3 ml	0	5 min, 37°C	600
Triglyceride	3 µl	260 µl	150 µl	5 min, 37°C per reaction	600/700
Free fatty acids	50 µl	1 ml	2 ml	10 min, 37°C per reaction	550
LDL cholesterol	3 µl	270 µl	90 µl	5 min, 37°C per reaction	600/700
HDL cholesterol	3 µl	270 µl	90 µl	5 min, 37°C per reaction	600/700

[a]Reaction volumes can be scaled to meet the sensitivity and/or volume capacity of spectrophotometer (e.g., 96-well microtiter plate reader).

1. Reconstitute the lipid analysis kit reagents according to the manufacturer's instructions.

   Color reagents are generally stable for ~1 month following reconstitution, when stored at 2° to 10°C.

2. Prepare standards by serially diluting the stock standard solution provided at 1:1, 1:2, 1:4, and 1:8.

   This should give final concentrations that cover both the lower and upper range of the linear curve. According to many manufacturers, the use of a single standard per reaction is generally sufficient after a calibration/standard curve has been established once to ensure linearity.

3. Pipet sample and standards into the appropriate tubes using the volumes indicated in Table 29B.2.1. Include a tube for a blank (deionized water).

4. Add the required amount of reagent #1 per tube and vortex. Incubate tubes for the indicated amount of time (Table 29B.2.1).

   Skip step 5 and proceed to step 6 if the kit involves only a single reagent.

5. Add the required amount of reagent #2 per tube and vortex. Incubate tubes for the indicated amount of time (Table 29B.2.1).

6. Measure the absorbance of each sample (A_s) and standard (A_{std}) against the blank at the suggested wavelength(s) A_1 and A_2 (Table 29B.2.1).

7. Prepare a standard curve by plotting absorbance versus concentration for the standard. Generate an equation of the line using linear regression.

8. Calculate amount of lipid for the sample (in mg/dl) as follows:

   a. *From a standard curve:* Read the concentration corresponding to the absorbance of the sample (A_s) from the calibration curve or calculate using the equation of the line $y = mx + b$, where m = slope of the curve and b = the x intercept.

   b. *From a single standard:* concentration of sample (in mg/dl) = $(A_s/A_{std}) \times$ concentration of standard (in mg/dl).

   c. *For dual-wavelength reactions:* Substitute A_s for ΔA_s $[(A_{2s} - A_{2blank}) - (A_{1s} - A_{1blank})]$ and A_{std} for ΔA_{std} $[(A_{2std} - A_{2blank}) - (A_{1std} - A_{1blank})]$.

9. Check results against the manufacturer's specifications of the linear range for the reaction.

For each parameter, a linear range is defined by the kit manufacturer. When the concentration of the parameter is outside this linear range, the results cannot be calculated accurately. If samples exceed the upper limit, they should be diluted with saline or water and the assay should be repeated. The result is then multiplied by the dilution factor. The lowest detectable level has also been estimated for each parameter; it represents the lowest measurable level of the parameter that can be distinguished from zero. It is calculated as the absolute mean plus three standard deviations of twenty replicates of an analyte-free sample.

ALTERNATE PROTOCOL 1

BLOOD LIPID ANALYSIS USING AUTOMATED LABORATORY WORKSTATION

Automated workstations are used increasingly for routine clinical chemistry evaluations in the mouse. Such workstations increase throughput and often reduce the sample volume required for analysis, because many measurements can be combined. Lipid analyses have been adapted for such an automated, high-throughput approach. For a comparison of automated lipid tests, see Table 29B.2.2.

Automated, high-throughput workstations are often found in laboratories that perform many assays, such as large clinical chemistry laboratories in veterinary and mouse clinics. Such high-throughput laboratories can often accommodate samples from outside laboratories. It is worthwhile to identify several laboratories with automated workstations and compare the price and quality of the services provided. One important consideration when opting to outsource tests is to verify that the tests were validated for mouse samples. Although routine cholesterol, triglyceride, and fatty acid measurements are interchangeable between species, differences exist for HDL and LDL cholesterol measurements, which often use species-specific antibodies.

This protocol uses an Olympus AU400 automated workstation, which is at present among the workstations that require the lowest sample volumes—an important advantage when analyzing mouse samples. This system is capable of analyzing serum, plasma, and urine samples, and measures the components of the samples optically. It automates the main processes, including dispensing of sample and reagent, mixing, and photometry. First, the sample probe dispenses serum or plasma in a sample cup that is set on a rack into the specified cuvette of the cuvette wheel unit (88 cuvettes are mounted on the cuvette wheel). Then the specific reagent, which has been added to the reagent refrigeration unit, is added to the sample by the reagent probe. The sample probe and reagent probe units

Table 29B.2.2 Comparison of Automated Lipid Tests

Test	Test principle	Units	Sample volume	Manufacturer/model
Cholesterol	Enzymatic color test, CHOD-PAP method	mmol/liter	3 µl	Olympus OSR 6116
HDL cholesterol	Immunoinhibition, enzymatic color test	mmol/liter	2 µl	Olympus OSR 6187
LDL cholesterol	Enzymatic color test, CHOP-PAP method	mmol/liter	3 µl	Olympus OSR 6183
Triglyceride	Enzymatic color test, GPO-PAP method	mmol/liter	3 µl	Olympus OSR 6133
Nonesterified fatty acids (NEFA)	Enzymatic color test, AcylCoA-synthetase + ATP	mmol/liter	8 µl	Wako

are equipped with a wash well for washing the probe after each sample is dispensed. The sample and reagent are mixed in the cuvette by a mixing device, and the system measures the absorbance of the sample. The system is equipped with a photometer lamp to optically measure the mixture in a cuvette. Finally, the optical density of the sample is calculated according to the absorbance measured. The data processor unit employs a personal computer to process data.

Calibration frequency using the Olympus calibrators depends on the parameters to be measured and the workflow. For most parameters described here, calibration is performed once every 5 days or when the analyzer requests a new calibration, or when the control value is outside the acceptable range. Calibrators are typically used undiluted to generate a single reference value, although manufacturer's instructions need to be followed in each case.

Materials

Calibrators for biochemistry on AU400:
- Olympus OE 66300 for cholesterol and triglyceride (lyophilized human serum with chemical additives and appropriate enzymes of human, animal, and plant origin)
- Olympus ODC0011 for HDL cholesterol and ODC0012 for LDL cholesterol (lyophilized human serum containing human HDL or LDL cholesterol)
- Wako 994-75409 for FFA

Intralaboratory controls for biochemistry on AU400:
- ODC0003 (Level 1) and ODC0004 (Level 2) Olympus System Reagent Control Serum (lyophilized preparation of stabilized human serum with adjusted levels of defined analytes)
- ODC0005 LDL/HDL Cholesterol Control Serum, Levels 1/2 (lyophilized preparation of stabilized human serum with adjusted levels of defined analytes)

Interlaboratory controls: Lyphochek unassayed chemistry control, human, levels 1 and 2 (Bio-Rad C-320-10 and C-325-10)

Serum or plasma samples (80 µl minimum)

Olympus AU400 automated laboratory workstation and operating manual (*http://www.olympus-global.com*) with appropriate sample tubes and software

Unity Plus quality control software (Bio-Rad)

4-ml, 50 × 14–mm hemolysis tubes (Sarstedt) with bar-coded labels added in-house

NOTE: For general operation of the analyzer, refer to the operator manual of the AU400.

1. Use the yellow rack for calibration and load the blue rack at position 1 with deionized water to determine the blank value.
2. Test all parameters daily with intralaboratory control sera (Olympus ODC0003, ODC0004, and ODC0005), which consist of lyophilized human plasma with normal and pathological lipid concentrations.

   a. Thaw and vortex the control sera before use.

   b. Load controls according to the analyzer's display.

   c. Manage all control data using software provided with Olympus automated workstation.

   Control values must lie within three standard deviations, otherwise the measurement will have to be repeated following recalibration.

3. Test all parameters daily with interlaboratory control sera (Bio-Rad level 1 and 2). Send data monthly to Bio-Rad using Bio-Rad Unity Plus software to provide

for an interlaboratory quality control assessment by the Bio-Rad External Quality Assurance Services.

This program provides a monthly report analysis.

4. Thaw the frozen unknown plasma samples, if needed. If samples are not available in sufficient quantity and/or are very lipemic, dilute 1:2 with deionized water.
5. Aliquot samples into tubes (provided by Olympus; alternatively, use microcentrifuge tubes with lids removed), then place into matching bar-coded hemolysis tubes (bar coding added in-house). Vortex all samples.
6. Load the racks according to the working lists, starting with rack #1. Perform the analyses.
7. Calculate values from the appropriate calibrations.

   Each parameter has a limit value specified by the manufacturer. The reaction is valid only within the limits of linearity. Results are generally reported as mmol/liter.

BASIC PROTOCOL 2

LIPOPROTEIN FRACTIONATION BY DIFFERENTIAL ULTRACENTRIFUGATION

When abnormalities are detected in total, HDL, or LDL cholesterol levels, it is useful to isolate and analyze the different fractions of lipoproteins. Two major techniques are used for the separation of lipoprotein fractions, differential ultracentrifugation and chromatography (see Alternate Protocol 2). Differential ultracentrifugation is a preparative method for separating lipoproteins on the basis of their hydrated density. At solvent densities higher than the lipoprotein density, lipoproteins float at a rate dependent on their density, size, and shape. The lipoprotein classes are sequentially isolated by stepwise increases in the solvent density through the addition of salts such as KBr, NaBr, or NaCl (see Fig. 29B.2.1), over a broad range of densities unequally distributed along a gradient from 0.92 to 1.25 g/ml (De Lalla and Gofman, 1954). The classification of lipoprotein particles according to buoyant density (d) includes: chylomicrons ($d < 0.94$ g/ml); very-low-density lipoproteins (VLDL; $d = 0.94$ to 1.006 g/ml); intermediate-density lipoproteins (IDL; $d = 1.006$ to 1.019 g/ml); low-density lipoproteins (LDL; $d = 1.019$ to 1.063 g/ml); and high-density lipoproteins (HDL; $d = 1.063$ to 1.21 g/ml). Their equivalent flotation rates (Sf) by analytical ultracentrifugation, at a background density of 1.063 g/ml, are >400 for chylomicrons, 20 to 400 for VLDL, 12 to 20 for IDL, and 1 to 12 for LDL. At a background density of 1.2 g/ml, HDL_2 and HDL_3 display flotation rates of 3.5 to 9 and 0 to 3.5, respectively. Once lipoprotein fractions are isolated, the particle content of cholesterol, TG, and different apolipoproteins can then be measured.

Materials

Serum or plasma: preferably 400 µl of freshly collected sample (samples collected under the same conditions can be pooled)
Density gradient solutions (see recipe)
Potassium bromide, solid
Dialysis buffer (see recipe)
1.0-ml polycarbonate ultracentrifuge tubes (Beckman)
Optima TL-100 ultracentrifuge with TLA-100.2 rotor (Beckman) or equivalent ultracentrifuge and rotor
Digital Density Meter DMA3.5 (Anton Paar; *http://www.anton-paar.com*)
Dialysis tubing (Spectra/Por membrane 3.5, Spectrum)
Beaker for dialysis
Additional reagents and equipment for dialysis (*APPENDIX 3C*), lipid assays (Basic Protocol 1), and protein quantitation (Support Protocol)

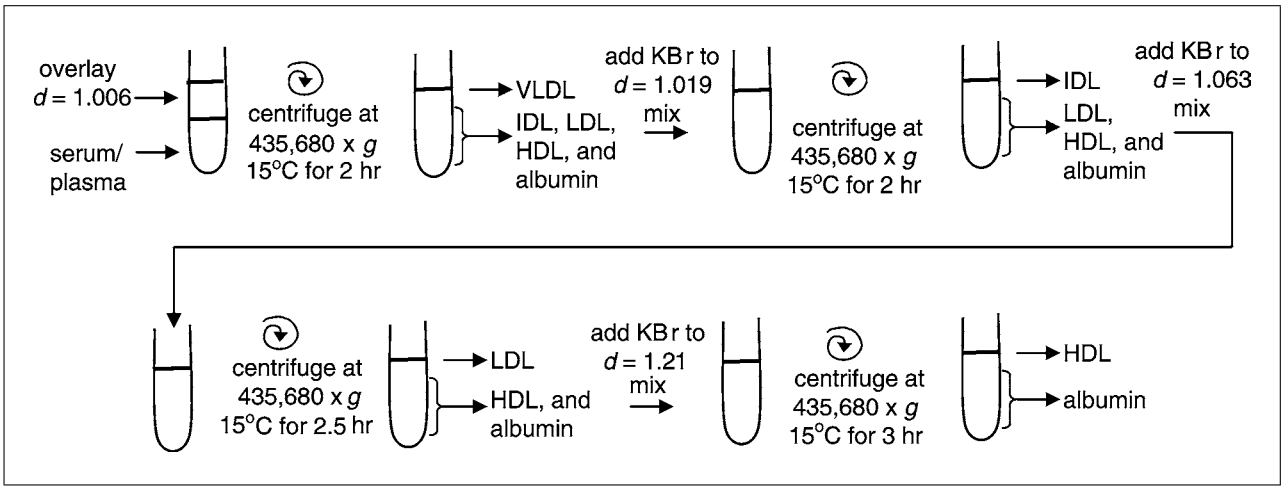


Figure 29B.2.1 Isolation of lipoprotein fractions by sequential ultracentrifugation.

Isolate VLDL (d = 1.006)

1. Aliquot 400 µl of serum into a 1-ml polycarbonate ultracentrifuge tube and overlay with 600 µl of the $d = 1.006$ density gradient solution.

2. Ultracentrifuge 2 hr at 435,680 × *g*, 15°C, without braking.

   All centrifugations in this protocol should be done with a slow deceleration rate, as braking could disrupt the phase separation.

3. Allow the centrifuged sample to return to room temperature and transfer the top layer (~400 µl), corresponding to VLDL, to a clean tube using a pipet. Set the bottom fraction aside on ice for continued fractionation.

   In general the top layer is an off-white color and can be distinguished from the bottom fraction, which contains the rest of the lipoproteins.

   IMPORTANT NOTE: *If the donor animals are not fasted, this top layer will contain chylomicrons as well. Chylomicrons are difficult to separate completely from VLDL; however, if the sample is centrifuged initially for 20 min, the supernatant should contain the larger and lighter particles resembling chylomicrons. The bottom layer is then processed again, as in steps 1 to 3, to isolate the VLDL.*

4. Prepare dialysis tubing by presoaking it in dialysis buffer (*APPENDIX 3C*). Tie a double knot at one end of the dialysis tubing, then add the VLDL fraction. Double-knot the other end close to the top of the solution to minimize the dilution of the sample. Place the dialysis tubing into a beaker and dialyze against 50 ml dialysis buffer for 24 hr at 4°C with constant mixing, changing the dialysis solution at least three times over this time interval.

5. Collect the dialyzed VLDL sample into a microcentrifuge tube. Set aside for lipid analysis.

Isolate IDL (d = 1.019)

6. Resuspend the 600 µl of bottom fraction in a polycarbonate tube and adjust the density from 1.006 to 1.019 by adding 24.3 µl of $d = 1.34$ density gradient solution.

   This volume is determined according to the formula $(A \times Y) + (B \times X) = (A + B) \times X$, where A = volume of sample (600 µl), B = volume of salt solution to add (in this case 24.3 µl), Y = the initial density (d = 1.006), Z = density of added salt solution (d = 1.34), and X = the final density (d = 1.019).

7. Adjust the sample to a final volume of 1 ml by adding $d = 1.019$ density gradient solution, and mix thoroughly.

8. Ultracentrifuge the sample 2 hr at 435,680 × *g*, 15°C, without braking.

9. Warm the centrifuged sample to room temperature and transfer the top layer (~400 µl), corresponding to IDL, to a clean tube using a pipet. Set the bottom fraction aside on ice for continued fractionation.

10. Dialyze as in steps 4 and 5.

Isolate LDL (d = 1.063)

11. Resuspend the bottom fraction (~400 µl) in a polycarbonate tube, add 95.3 µl of $d = 1.34$ density gradient solution, and bring the volume to 1 ml with $d = 1.063$ density gradient solution. Mix, then ultracentrifuge 2.5 hr at 435,680 × *g*, 15°C.

12. Allow the sample to return to room temperature and transfer the top layer (~400 µl), corresponding to LDL, to a clean tube using a pipet. Set the bottom fraction aside on ice for continued fractionation.

13. Dialyze as in steps 4 and 5.

Isolate HDL (d = 1.21)

14. Resuspend the bottom fraction (600 µl) in a microcentrifuge tube. Adjust the density to 1.21 by adding 678.5 µl of $d = 1.34$ solution, and add 21.5 µl of $d = 1.21$ density gradient solution to bring the sample to a final volume of 1.3 ml.

15. Mix the sample, then transfer 1 ml to a polycarbonate tube and ultracentrifuge 3 hr at 435,680 × *g*, 15°C.

16. Allow the sample to return to room temperature and transfer the top layer (~400 µl), corresponding to HDL, to a clean tube using a pipet.

    The bottom fraction corresponds to the lipoprotein-depleted plasma and can also be analyzed as a control if desired.

17. Dialyze as in steps 4 and 5.

Analyze fractions

18. Analyze dialyzed fractions for total cholesterol and TG content (see Basic Protocol 1) and/or protein content (see Support Protocol).

    In general, TG are the predominant lipids in the VLDL fraction, but are very low in the LDL fraction.

    To determine the concentration of the various analytes in the HDL fraction, a correction factor of 1.3 is necessary. Lipid content can be normalized to the volume of initial serum to give mg/dl, or to the protein content to give mg lipid/µg protein.

ALTERNATE PROTOCOL 2

LIPOPROTEIN ANALYSIS BY GEL-EXCLUSION CHROMATOGRAPHY

Separation of lipoprotein fractions can also be performed by gel filtration, which separates proteins on the basis of particle size (Brousseau et al., 1993). Briefly, the plasma samples are applied to a column and eluted at a constant flow rate using elution buffer. The eluted sample is then passed through an online UV detector prior to collection with a fraction collector. The fractionated samples are then subjected to manual or automated lipid measurements (e.g., cholesterol and TG) using enzymatic reagents as in Basic Protocol 1. A profile is then obtained that partitions the amount of lipid or protein according to the different lipoprotein fractions, which can be identified by their known retention times (VLDL = 33 min, LDL = 38 min, and HDL = 47 min).

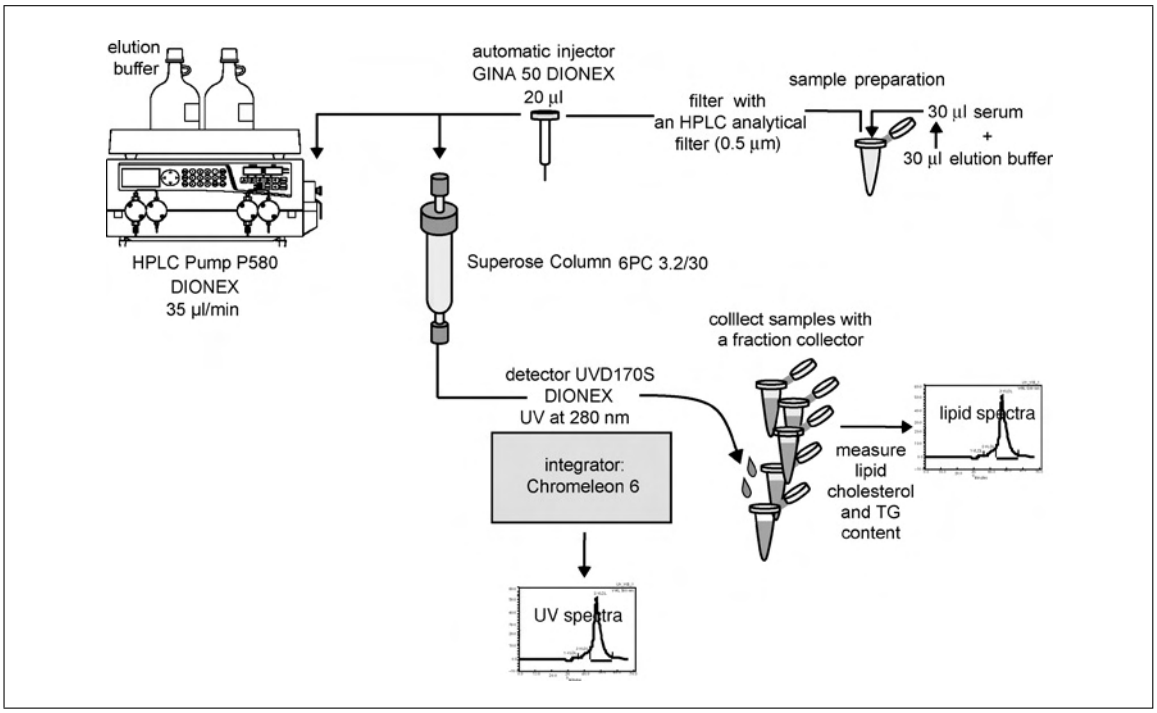

elution buffer
automatic injector GINA 50 DIONEX 20 µl
sample preparation
filter with an HPLC analytical filter (0.5 µm)
30 µl serum
+
30 µl elution buffer
HPLC Pump P580 DIONEX 35 µl/min
Superose Column 6PC 3.2/30
colllect samples with a fraction collector
detector UVD170S DIONEX UV at 280 nm
lipid spectra
integrator: Chromeleon 6
measure lipid cholesterol and TG content
UV spectra

Figure 29B.2.2 FPLC schematic diagram.

Materials

Serum or plasma
Elution buffer (see recipe)
20% (v/v) ethanol
FPLC system (Fig. 29B.2.2) consisting of:
HPLC pump (P580 A, Dionex)
Multi-sample automatic injector (GINA 50, Dionex)
Column (Superose 6 PC 3.2/30, Amersham Biosciences)
UV/VIS detector (UV170S, Dionex)
Fraction collector (FRAC-100, Amersham Biosciences)
Integrator software (Chromeleon 6.4, Dionex)
0.22- or 0.45-µm HPLC analytical filter for Biocompatible UPCA
Additional reagents and equipment for lipid assays (Basic Protocol 1)

1. Set up an FPLC-type unit as shown in Figure 29B.2.2.
2. On day 1 of the experiment, install the Superose column according to the manufacturer's instructions. To equilibrate the column for first-time use or after long-term storage, flush out the storage solution (generally 20% ethanol) with 2.4 ml water at a flow rate of 20 µl/min, then equilibrate the column with elution buffer at a flow rate of 40 µl/min. Continue until the system is completely free of air bubbles and the UV signal is stable at the selected sensitivity (minimum of 6 hr, preferably overnight).
3. On day 2 of the experiment, prepare the samples by mixing 30 µl of plasma or serum (preferably plasma) with 30 µl of elution buffer. Filter the samples with either a 0.45- or 0.22-µm filter to remove aggregates.

   The maximum protein capacity for the column is ~0.5 to 3 mg in a maximum concentration of 50 mg/ml. This range maximizes the resolution and detection of the protein.
4. Place the samples in the autoinjector and set the machine to inject 20 µl of sample onto the column.

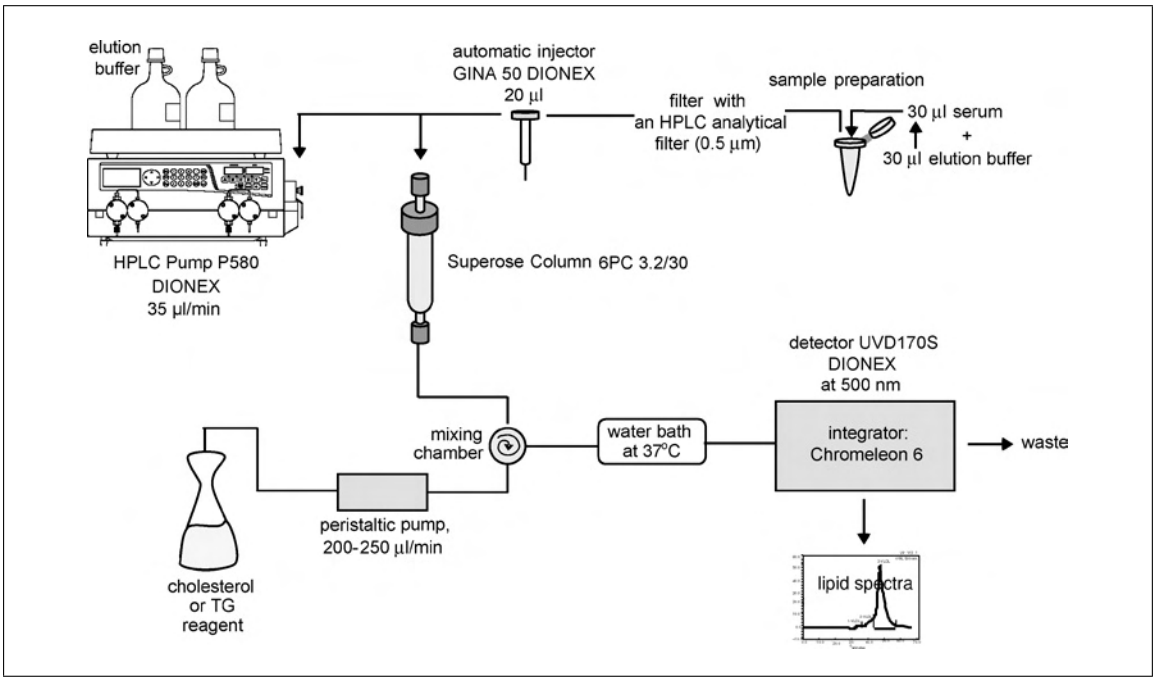


Figure 29B.2.3 Schema showing the adaptation of FPLC to include online lipid measurements.

5. Inject the first sample and pass elution buffer through the column at a flow rate of 35 µl/min. Use an online fraction collector to collect ~30 to 40 fractions of 75 to 150 µl in microcentrifuge tubes. Measure A_{280} with the online UV detector and record the values using the Chromeleon Integrator system.

   In general, the first few fractions can be discarded. Total separation time per sample is 70 min. The known retention times are 33 min for VLDL, 38 min for LDL, and 47 min for HDL.

6. Prior to separating the next sample, wash column with elution buffer at a flow rate of 35 µl/min for 10 min.

7. Measure the amount of cholesterol and TG in each fraction as described in Basic Protocol 1.

8. Integrate data into a lipoprotein profile using the Chromeleon software according to the manufacturer's instructions, and/or by plotting the lipid content according to fraction number or A_{280} measurement.

   One possible adaptation of the FPLC technique described above is to include the capacity for online lipid measurements. A schema showing this adaptation is shown in Figure 29B.2.3; however, specific details are beyond the scope of this unit.

9. At the end of the experiment, wash the column sequentially with 4.8 ml water and 4.8 ml 20% ethanol at a flow rate of 35 µl/min. Check that the UV signal is stable after each wash. To prevent drainage during storage, always fill the empty space above the filters and the rubber tubing with 20% ethanol before replacing the protective caps. Store the columns at 4°C.

   Columns can be reused up to 100 times. See manufacturer's instructions for special column procedures and troubleshooting if there is a color change in the gel bed, a loss of resolution, or decreased sample recoveries.

TISSUE LIPID ANALYSIS

Lipids can be extracted from tissue (frozen or fresh) and partitioned according to type by using the method of Folch et al. (1951), which is based on a chloroform/methanol extraction. The quantity of cholesterol and TG in the tissue extracts can then be determined by the same enzymatic assays used to detect these lipids in serum. In some instances, it is of interest to distinguish between cholesterol that is esterified and free. Cholesterol ester (CE) represents the metabolically inert pool of cholesterol and is mainly found in the storage lipid droplets, as opposed to free cholesterol, which is associated with the membrane as well as with metabolic regulation. In order to obtain a measurement of CE, enzymatic assays that detect total cholesterol (esterified plus nonesterified) are used in combination with enzymatic assays that detect just free cholesterol. CE is then determined by the subtraction of the free cholesterol measurement from the total cholesterol value.

In general, the lipids that are extracted by chloroform/methanol can be dissolved in 95% ethanol or isopropanol and then quantified via the enzymatic assays. Alternatively, the chloroform/methanol extracts can be dissolved in a 1% Triton X-100 solution in chloroform; the chloroform is then evaporated away, with water being added as the final solvent prior to the measurement of the lipid content by enzymatic assay (Carr et al., 1993). This latter method can be advantageous, as some enzymatic reagents are not compatible with ethanol, and ethanol is a very volatile solvent, making it difficult to manipulate accurately.

CAUTION: Plastic tubes should be avoided due to the possibility of solvents leaching out contaminating lipids. Avoid evaporation of solvents by applying caps or corks to tubes.

Materials

Tissue (~0.5 g)
2:1 (v/v) chloroform/methanol
Methanol
1α,2α-(n)-[^{3}H]Cholesteryl oleate (Amersham Biosciences)
Glycerol tri[1-^{14}C]oleate (Amersham Biosciences)
Chloroform
0.73% (w/v) NaCl
Chloroform/methanol/NaCl (see recipe)
95% ethanol *or* 1% (v/v) Triton X-100 in chloroform
200 μg/ml cholesterol/triglyceride standard solution: dilute cholesterol and triolein to a final concentration of 200 μg/ml in isopropanol
14 × 100–mm Pyrex or borosilicate glass round-bottom tubes with screw caps
Homogenizer (Polytron or equivalent)
12 × 75–mm glass hemolysis tubes with corks
Whatman filter paper (lipid free)
Nitrogen evaporator with dry bath at ~50°C (e.g., N-EVAP from Organomation)
Scintillation vials, scintillation fluid, and β-counter with dual-channel program for ^{3}H and ^{14}C

Extract tissue

1. Obtain a piece of tissue with a wet weight of ~0.5 g and place in a Pyrex round-bottom tube. Add 900 μl of 2:1 chloroform/methanol and homogenize for ~30 sec at high speed in a Polytron-type homogenizer.
2. Add 300 μl of methanol and vortex. Add 2 μl each of 1α,2α-(n)-[^{3}H]cholesteryl oleate and glycerol tri[1-^{14}C]oleate to the sample. Also add the same amounts of these compounds to a scintillation vial to determine counts added.
3. Cap tubes and centrifuge 15 min at 750 × *g*, room temperature.

4. Using a glass Pasteur pipet, remove the supernatant to a 12 × 75–mm glass hemolysis tube. If pieces of tissue still contaminate the sample, filter the sample into a new tube using lipid-free Whatman filter paper.
5. Add 400 μl chloroform and vortex.
6. Add 275 μl of 0.73% NaCl and vortex vigorously for ~30 sec.
7. Centrifuge samples 3 min at 750 × *g*, room temperature.

   A phase separation should be visible.
8. Using a Pasteur pipet, remove the top phase and discard. Wash the phase interface by carefully adding 800 μl chloroform/methanol/NaCl with a Pasteur pipet, taking care not to mix the phases. Remove and discard the top phase again and wash the interface another two times in the same manner.
9. While vortexing, add a sufficient amount of methanol to the bottom phase until a single phase is obtained.
10. Evaporate the sample to dryness under a steady stream of nitrogen in a nitrogen evaporator with ~50°C dry bath.
11. Add 500 μl of 95% ethanol or 1 ml of 1% Triton X-100 in chloroform. Vortex, then evaporate to dryness as in the previous step. Resuspend sample in 500 μl deionized water.

Perform lipid assay

12. Generate a standard curve to be used in the enzymatic lipid assays, by preparing duplicate aliquots of 0 (blank), 5, 10, 20, 40, 60, 80, and 160 μl of 200 μg/ml cholesterol/triglyceride standard solution.
13. Evaporate the standards to dryness under nitrogen as in step 10. Resuspend in 500 μl of 95% ethanol or in 1 ml of 1% Triton X-100 in chloroform. For the standards in 1% Triton X-100/chloroform, dry down again and resuspend in 500 μl of deionized water.
14. Determine total cholesterol, free cholesterol, and/or TG content as in Basic Protocol 1. Retain a 25-μl aliquot of each sample and determine radioactivity by β counting.
15. Calculate the percent recovery for cholesterol and TG by dividing the number of counts extracted in the sample by the total number of counts added (step 2), and multiplying the result by 100.

    % recovery for cholesterol = (^{3}H dpm of sample/total ^{3}H dpm added) × 100%
    % recovery for TG = (^{14}C dpm of sample/total ^{14}C dpm added) × 100%.
16. Adjust the total cholesterol, free cholesterol, and TG (step 14) by multiplying the observed values (in μg lipid/mg tissue) by the percent recovery.
17. Determine the amount of cholesterol ester (CE in μg/mg tissue) by subtracting the amount of free cholesterol from total cholesterol.

BASIC PROTOCOL 4

ENZYMATIC SERUM BILE ACID MEASUREMENT

Bile acids constitute a group of structurally different molecules synthesized in the liver as a breakdown product of cholesterol (Houten and Auwerx, 2004). In humans, these include the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA), their glycine and taurine conjugates, and the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA). In mice, CDCA and glycine conjugates are practically absent. Instead, muricholic acid (MCA), which is derived from CDCA, is prominent. Since

bile acid synthesis, secretion, extraction from portal blood, and re-excretion all depend on adequate liver function, quantification of serum bile acids represents a sensitive and specific test for liver function. Even more important in the context of metabolic phenotyping is the quantification of bile acid pool size, as most of the regulation of hepatic cholesterol metabolism by bile acids is attributed to the size of the bile acid pool. This is exemplified by the negative feedback mechanism by which bile acids returning to the liver control their own synthesis. However, since recent evidence suggests that the regulatory nature of the bile acid pool depends not just on its size but also on composition, the measurement of both bile acid pool size and composition (see Alternate Protocol 4) are the ultimate goal for evaluating the effect of bile acids (Houten and Auwerx, 2004). For example, a higher abundance of hydrophilic bile acids within the pool seems to induce a greater biliary lipid secretion, whereas hydrophobic bile acids seem to be less effective (Einarsson and Grundy, 1980).

In general, serum bile acids are evaluated under fasting and post-prandial conditions and can be measured using simple enzymatic colorimetric assays (manual or with an automated workstation), or more elaborate methods such as radioimmunoassays or tandem mass spectrometry (see Alternate Protocol 3; Setchell and Matsui, 1983). All bile acids contain a 3α-hydroxyl group, which can be converted to a 3-keto group by 3α-hydroxysteroid dehydrogenase. This enzyme simultaneously converts NAD^+ to NADH, which can be measured spectrophotometrically using different approaches. For example, NADH can be measured by the conversion of nitroblue tetrazolium (NBT) to the formazan dye catalyzed by diaphorase (Mashige et al., 1978).

Materials

Colorimetric Total Bile Acids Assay Kit (containing reagents R1, R2, and R3; Bio-Quant, *http://www.bio-quant.com*)
10 mM taurocholic acid
Bile acid control/calibrator (high and low level; Diagnostics Chemical Limited, *http://www.dclchem.com*)
200-µl cuvettes
Spectrophotometer

1. Reconstitute the contents of one bottle of R3 with 10 ml of reconstitution buffer (R1). Note the date of reconstitution and the expiration date as indicated by the manufacturer.
2. Prewarm R2 and the reconstituted R3 to room temperature.
3. Serially dilute 10 mM taurocholic acid with water so that the dilutions cover the upper and lower limits of the linear range (1 to 200 µM).

   The method is linear up to 200 µM. Samples above this range should be adjusted by dilution with saline or water. This self-made taurocholic acid standard can be compared with the single standard of 35 µM from Bio-Quant.

4. To a 200-µl cuvette, add 150 µl of reconstituted R3 and 20 µl of sample, standard, quality control/calibrator (high and low level of bile acid), or blank (water). Mix well and incubate for 4 min at 37°C.
5. Add 30 µl of R2, vortex, and immediately read the absorbance at 540 nm as A_1.
6. Incubate 5 min and read the absorbance at 540 nm as A_2.
7. Calculate ΔA for each sample and standard using the formula:

   $$\Delta A = (A_2 - A_1).$$

8. Generate a standard curve by plotting absorbance versus concentration. Use linear regression to determine the total bile acid concentration of the sample.

   Alternatively, the total bile acid concentration can be determined using a single standard (e.g., 35 μM) and the formula:

   total bile acids (in μM) = $\Delta A_{sample}/\Delta A_{standard} \times$ standard (in μM).

   However, a standard curve should be used instead of a single standard to ensure linearity of the reaction.

ALTERNATE PROTOCOL 3

SERUM BILE ACID MEASUREMENT BY HPLC TANDEM MASS SPECTROMETRY

In order to discriminate between the different bile acid molecules, a reversed-phase HPLC separation of serum can be coupled to the enzymatic detection of bile acids. For this, however, relative large volumes of plasma are needed because of the relatively low sensitivity of the enzymatic assay, which makes this somewhat impractical for measurements in the mouse. Analysis with tandem mass spectrometry (MS/MS) has many advantages. Most importantly, MS/MS gives specific structural information, which in combination with HPLC separation can unambiguously confirm the identity of a molecule. In addition, MS/MS analysis has higher sensitivity when compared with enzymatic detection methods. The sensitivity depends on the type of MS/MS used; in our method the detection limit is 0.05 μmol/liter for conjugated bile acids. MS/MS analysis is thus considered the "gold standard" for analysis of bile acids. A disadvantage is that it requires specific equipment and the availability of stable isotope-labeled standards.

Materials

Plasma
Internal deuterium-labeled standards:
  Chenodeoxycholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-2772)
  Cholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-2452)
  Glycochenodeoxycholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-5673)
  Glycocholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-3878)
  Taurine conjugates (custom synthesized)
Acetonitrile (HPLC grade)
1:3 (v/v) methanol/water
1.5-ml microcentrifuge tubes
4-ml glass vials
Nitrogen evaporator with dry bath at ~40°C (e.g., N-EVAP from Organomation)
HPLC-MS/MS (e.g., Hewlett-Packard HP1100 binary pump and a Waters Micromass Quatro II tandem mass spectrometer equipped with electrospray ionization)
Additional reagents and equipment for liquid chromatography–mass spectrometry (*UNIT 10.21*)

1. Premix the internal standards in water to prepare a cocktail containing 10 μM of each internal standard. Add 20 μl of this cocktail (200 pmol of each standard) to 20 μl of plasma in a 1.5-ml microcentrifuge tube.
2. Add 250 μl of acetonitrile and mix immediately to precipitate the proteins.
3. Microcentrifuge 12 min at 12,000 × *g*, 4°C.
4. Decant supernatant into a 4-ml glass vial.
5. Evaporate the solvent under a stream of N_2 with heating at 40°C.
6. Dissolve the residue in 70 μl of 1:3 methanol/water.

7. Analyze the samples by HPLC-MS/MS (*UNIT 10.21*).
8. Calculate the concentration of bile acids (in μM) by comparing the labeled stable isotopes with the specific bile acids analyzed.

BILE ACID POOL SIZE AND COMPOSITION

ALTERNATE PROTOCOL 4

The bile acid pool is composed of all bile acids that are present in the liver, gallbladder, and small intestine. As only a small amount of bile acids circulate at a given time in the serum, serum bile acid levels and composition do not always accurately reflect bile acid homeostasis. For this reason, information on the bile acid pool, including of its size and relative composition, yields important additional information about whole-body cholesterol and bile acid homeostasis. Although bile acid pool size can be determined using enzymatic assays and/or radioimmunoassays, the authors recommend using HPLC-MS/MS, since this also reveals the exact composition of the bile acids present in the bile acid pool.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Test animal of interest
100% ethanol (HPLC grade)
Recovery standard (see recipe)
Internal deuterium-labeled standards:
  Chenodeoxycholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-2772)
  Cholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-2452)
  Glycochenodeoxycholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-5673)
  Glycocholic-2,2,4,4-d_4 acid (C/D/N Isotopes cat. no. D-3878)
  Taurine conjugates (custom synthesized)
Dissecting instruments
50-ml conical polypropylene centrifuge tubes (e.g., Falcon)
50-ml glass beaker
Watch glass suitable as cover for 50-ml beaker
Filter paper (Schleicher-Schuell, type 597)
Glass funnels
50-ml volumetric flask
HPLC-MS/MS (e.g., Hewlett-Packard HP1100 binary pump and a Waters Micromass Quatro II tandem mass spectrometer equipped with electrospray ionization)
Additional reagents and equipment for liquid chromatography–mass spectrometry (*UNIT 10.21*)

Extract bile acids

1. Remove gallbladder, liver, and the entire small intestine, with contents, from the animal. Store in a 50-ml conical polypropylene centrifuge tube at −80°C until use.

   Take extra care not to tear the small intestine, as any leakage of the contents will affect the quantitative measurement. This also applies to the gallbladder and its contents.

   It is not necessary to process the entire liver, since this organ normally contains only a small portion of the enterohepatic bile acids. Small pieces of liver can be removed and used for other methods, such as lipid or RNA extraction.

2. Transfer the organs to a 50-ml glass beaker, rinsing the storage tube twice with 100% ethanol. Cut tissues into small pieces (1-cm long) with scissors. Add a stir bar.

   The total volume of ethanol in the glass beaker should be ~40 ml.

3. Add exactly 1 ml of recovery standard to each sample.
4. Place the beaker on a hot plate/stirrer for extraction. Cover the beaker with a watch glass. Set the heat so that the ethanol comes to a gentle boil.
5. After 5 min of boiling, remove the beaker from the hot plate and allow to cool to room temperature.
6. Filter the sample using a glass funnel and filter paper into a 50-ml volumetric flask. Rinse beaker with 100% ethanol twice. Bring sample to a volume of 50 ml with 100% ethanol.

Perform assay

7. Transfer 1 ml of ethanolic extract to a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at 15,000 × *g*, room temperature.
8. Dilute supernatant 1:10 with water.
9. Premix the internal standards in water to prepare a cocktail containing 10 μM of each internal standard. Mix equal volumes of the diluted extract and internal standard cocktail (resulting mix contains 5 μM of each internal standard). Analyze the samples by HPLC-MS/MS (*UNIT 10.21*).
10. Calculate the concentration of bile acids (in μM) by comparing the labeled stable isotopes with the specific bile acids analyzed.
11. Calculate the amount of bile acids present in the enterohepatic tissues by correcting for dilution and the amount of norcholic acid added.
12. Divide the result by the body weight of the animal to calculate the amount of bile acids in μmol/kg.

SUPPORT PROTOCOL

MODIFIED LOWRY ASSAY FOR LIPOPROTEIN DETERMINATION

For the measurement of protein, several absorbance-based colorimetric methods exist. The most utilized methods rely on the reduction of copper in the presence of a chromogenic reagent. Alternatively, direct absorbance measurements of protein solutions at 280 nm can be performed (*UNIT 10.1A*). The latter technique is readily applied to online detection with FPLC-fractionated samples, whereas a modification of the Lowry procedure can be used for manual measurements. A method for the modified Lowry is provided here and readers are directed elsewhere for alternate methods (Markwell et al., 1978).

The two key modifications in this method include the addition of sodium dodecyl sulfate (SDS) and an increase in the concentration of copper tartrate reagent. The use of SDS in the alkali reagent enables the method to be used with membrane and lipoprotein samples without prior solubilization or lipid extraction. The overnight incubation in alkali is avoided, thus reducing the risk of autooxidation of the lipoprotein samples. The increase in copper tartrate reagent allows the assay of samples containing substantial amounts of sucrose or EDTA without pretreatment. Because precipitating protein quantitatively (i.e., with trichloroacetic acid) may be difficult and increases the time required, this method allows one to process large numbers of samples in a shorter period of time.

Materials

Lowry reagents A and B (see recipe)
Folin & Ciocalteau's 2N phenol reagent (e.g., Sigma)
1 mg/ml bovine serum albumin (BSA) standard solution (store at 4°C)
2 N NaOH
Spectrophotometer

1. On the day of the assay, prepare reagent 1 by mixing 1 part Lowry reagent B with 100 parts Lowry reagent A.
2. Also on the day of the assay, prepare reagent 2 by mixing Folin & Ciocalteu 2N phenol 1:1 with deionized water.
3. Generate standards by preparing duplicate aliquots of 0 (blank), 5, 10, 20, 40, 60, and 80 μl of 1 mg/ml BSA standard solution. Dilute each aliquot to 200 μl with water.

   The standard dilutions should cover the linear part of the standard curve.
4. Prepare duplicate aliquots of sample (for lipoproteins, generally 5 to 10 μl is sufficient). Dilute each aliquot to 200 μl with water.
5. Add 60 μl of 2 N NaOH to each tube and vortex.
6. Add 1 ml of reagent 1 to each tube, vortex, and let incubate for 10 min at room temperature.
7. Add 100 μl of reagent 2 to each tube, vortex, and let incubate for 1 hr at room temperature.
8. Measure the absorbance at 740 nm.
9. Generate a standard curve by plotting a graph of absorbance versus standard concentration. Generate an equation of the line using linear regression. Determine protein concentrations of the unknown samples by comparison with the standard curve.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Chloroform/methanol/NaCl

3 ml chloroform
48 ml methanol
47 ml 0.58% (w/v) NaCl
Store up to 3 months at 4°C

Density gradient solutions

Prepare the following stock salt solutions:

Solution A (d = 1.005): dissolve 8.766 g NaCl in 1 liter of aqueous 1% (w/v) disodium EDTA for final concentration of 0.15 M NaCl.

Solution B (d = 1.346): dissolve 153 g NaCl and 354 g KBr in 1 liter of aqueous 1% (w/v) disodium EDTA.

Generate all density solutions needed by diluting solution B ($d = 1.346$) with solution A ($d = 1.005$) according to the following equation:

$(A \times 1.005) + (B \times 1.346) = (A + B) \times X$

where A = volume of solution A ($d = 1.005$); B = volume of solution B ($d = 1.346$); and X is the desired density of the mixture.

For example, to make 12.05 ml of d = 1.063, add 10 ml of solution A and 2.05 ml of solution B. Densities of solutions should be verified by measurement on a Digital Density Meter DMA35.

Store solutions up to several months at room temperature.

Dialysis buffer

10.8 g $Na_2HPO_4 \cdot 12H_2O$
1.6 g $NaH_2PO_4 \cdot 2H_2O$
35.06 g NaCl
0.4 g NaN_3
0.4 g disodium EDTA
Deionized H_2O to 4 liters
Adjust pH to 7.4 with NaOH
Store up to several months at 4°C

Elution buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
30 mM NaCl
Degas and filter through 0.22-μm filter
Store up to several months at 4°C

Lowry reagents A and B

Lowry reagent A:
2% (w/v) Na_2CO_3
0.16% (w/v) sodium tartrate
1% (w/v) SDS
Store up to several months at room temperature
Lowry reagent B:
4% (w/v) $CuSO_4 \cdot 5H_2O$
Store up to several months at room temperature

Recovery standard

Dissolve 1 mg norcholic acid (23-nor-5β-cholanic acid-3α,7α,12α-triol) in 50 ml of 100% ethanol and adjust volume to 100 ml with Milli-Q water. Store up to 1 year at room temperature.

COMMENTARY

Background Information

Lipoproteins are supramolecular complexes consisting of lipids and proteins that function in the transport of nonpolar lipids from their sites of synthesis to their sites of utilization (Fig. 29B.2.4; Schaefer et al., 1978, Shepherd and Packard, 1989; Lee et al., 2003). These lipoproteins generally assume a spherical structure whereby the polar phospholipids, proteins, and unesterified or free cholesterol form a monolayer shell that facilitates the transport of core hydrophobic lipids, including cholesterol ester (CE) and triglycerides (TG), in the aqueous blood system. The nonpolar lipids serve numerous biological functions, acting as a transportable metabolic energy pool, as structural components for cell membranes, and as building blocks for hormones. The lipoprotein-associated proteins, or apolipoproteins (apo), have several functions including stabilizing lipid emulsions (apoA, B, C), acting as cofactors and modulators of enzymatic reactions (apoC, and apoA-I), managing the export of lipids out of cells (apoE and apoB), assisting in the clearance of lipids by target organs and cells through specific receptor interactions (apoB, apoE and apoA), and interfering with platelet aggregation (apoH; Alaupovic, 1991). In humans, abnormalities of lipoprotein transport, synthesis, and degradation, usually of genetic etiology, often lead to disease. Most notably, the metabolic syndrome has an important lipid component including increased plasma levels of TG, apoB, and small dense LDL particles, as well as reduced apoA-I and HDL cholesterol levels. Long-term dyslipoproteinemia associated with the metabolic syndrome increases the risk of atherosclerotic vascular disease and coronary artery disease (Grundy, 2004).

Three pathways can summarize the transport of lipoproteins: exogenous cholesterol transport, forward (endogenous) cholesterol transport, and reverse cholesterol transport (Shepherd and Packard, 1989; Lee et al.,

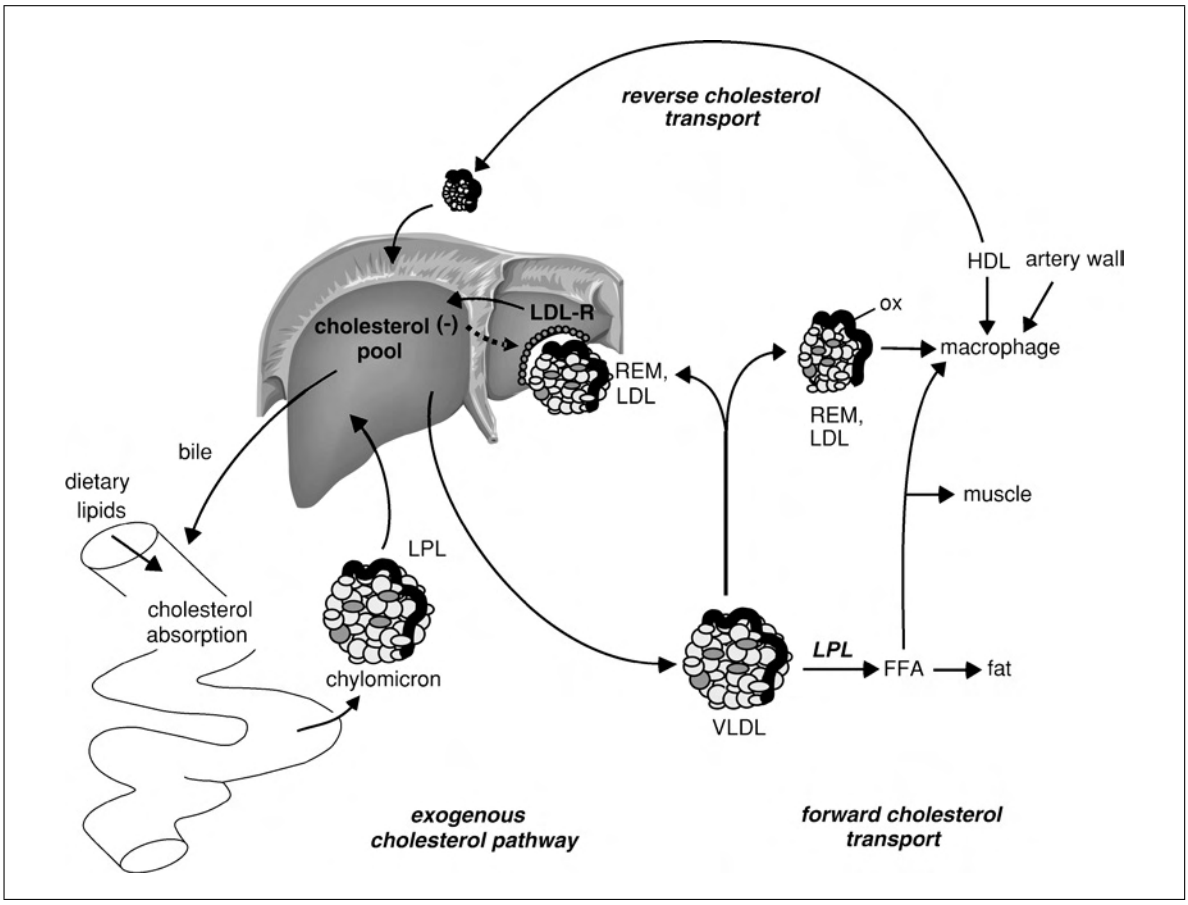


Figure 29B.2.4 A figure depicting the exogenous, forward, and reverse cholesterol pathways.

2003; Assmann and Gotto, 2004). The exogenous pathway describes the absorption and packaging of dietary-derived lipids into large TG-rich particles called chylomicrons. Chylomicrons are synthesized within the intestinal enterocytes and contain predominantly dietary-derived TGs. Lipoprotein lipase (LPL) hydrolyzes the core TG of the chylomicron, producing CE-rich chylomicron remnants, which are cleared by receptor-mediated endocytosis by the LDL receptor and/or remnant receptors. The forward cholesterol transport pathway involves the assembly and secretion of VLDL by the liver, replacing chylomicrons as the main vehicle of TG transport in the circulation in the post-absorptive state. VLDL are synthesized within hepatocytes and contain mainly endogenously derived TG. VLDL particles are acted upon by circulating lipases, including LPL, resulting in the release of free fatty acids (FFA). The CE-enriched particles—IDL or remnants and LDL—are subsequently cleared by LDL-R-mediated endocytosis in the liver or peripheral tissues including macrophages. Alternatively, these lipoproteins can be oxidized and cleared by macrophages via scavenger receptor–mediated endocytosis. Lipoprotein uptake by arterial macrophages promotes the formation of lipid-laden cells and consequently atherosclerosis.

The reverse cholesterol transport pathway functions to balance cholesterol deposition in the periphery through the receptor-mediated uptake and retention of apoB-containing lipoproteins. This pathway involves the hepatic secretion of nascent or lipid-poor HDL that serves to remove and carry free cholesterol from the extrahepatic tissues back to the liver, where HDL is degraded and cholesterol is excreted. The ATP-binding-cassette protein ABCA1 is one membrane protein implicated in the lipidation of these nascent HDL particles, with phospholipids thus generating HDL particles that can acquire cholesterol from membrane raft domains in the peripheral tissues. The subsequent exposure of these HDL particles to enzymatic and transfer activities in the plasma transforms them into mature spherical HDL particles designed for clearance by the liver. The interested reader is referred to an extensive review on cholesterol transport pathways (Shepherd and Packard, 1989; Lee et al., 2003; Assmann and Gotto, 2004).

Once transferred back to the liver through the reverse cholesterol transport pathway, excess cholesterol can be converted into bile acids or directly secreted into bile and eliminated from the body. As such, bile formation and bile acid biosynthesis have an important function in whole-body cholesterol homeostasis (Houten and Auwerx, 2004). Bile acids are essential for the absorption of dietary lipids, including fat-soluble vitamins. In recent years, an important role for bile acids as signaling molecules has emerged (Houten et al., 2006). Different bile acids may have different signaling capacities, underpinning the importance of determining bile acid composition and pool size. Most of the bile acids are present within the enterohepatic organs. Usually, bile acids are stored in the gallbladder; however, when a meal is ingested, they flow into the duodenum and intestine. The bile acids are efficiently (95%) absorbed again by passive diffusion and active transport from the terminal ileum, then transported back to the liver via the portal vein. The liver extracts bile acids efficiently from the portal vein and transports them back into the bile duct. From here they can go to the gallbladder for storage or flow into the intestine again. This completes their enterohepatic circulation.

In general, plasma lipoproteins exist in a dynamic equilibrium; thus, what is observed by analyzing lipoprotein fractions is really a static snapshot of this equation. It is best to evaluate lipid and lipoprotein homeostasis in the context of plasma lipid measurements combined with isolated lipoprotein determinations. For example, when there is an elevation in plasma TG in the chylomicron fraction, this may be interpreted as a disorder in chylomicron clearance subsequent to a problem with LPL-mediated hydrolysis of TGs in these chylomicrons. When TG levels are elevated together with elevations in plasma cholesterol and low HDL values, this may rather indicate the presence of the metabolic syndrome with insulin resistance. More detailed measurements, such as individual apolipoprotein content, are possible through such techniques as immunoassays and isoelectric focusing gel electrophoresis. However, these types of data are not clinically useful by themselves and should always be interpreted in the context of the plasma lipid profiles and lipoprotein fraction analysis. For example, the apoB level may provide information on the number of potentially atherogenic lipoproteins, as there is one apoB molecule per particle of chylomicron, VLDL, LDL, or IDL. In humans, the presence of small, dense LDL particles has been shown to be associated with the metabolic syndrome, and an elevated LDL apoB level may represent the presence of small dense LDL.

Finally, when analyzing lipids in mice, it is important to remember that mice are different from humans. Mouse HDL cholesterol represents between 65% and 75% of the total cholesterol, whereas the LDL cholesterol fraction is only between 10% and 20%. In contrast, human HDL represents less than 20% of the total cholesterol and HDL over 50% to 60%. This is in part due to differences in the metabolism of HDL. Mice have a significantly higher level of circulating lipases, lack the cholesterol ester transfer protein CETP, and have high levels of the phospholipid transfer protein PLTP (Tall, 1995). This is one of the reasons why it is often difficult to mimic human lipid disorders in a mouse. In order to circumvent such problems it is necessary to use transgenic mice, such as the apoE knockout, and/or challenges with environmental conditions, such as administration of a high-fat diet, which can result in more human-like lipoprotein-associated pathophysiologies.

Critical Parameters

Blood lipid parameters

To make an initial assessment of lipid and lipoprotein homeostasis in mouse models, the measurement of TG, FFA, and total, LDL, and HDL cholesterol comprise the minimal set of tests required. Blood lipid levels are sensitive to numerous parameters: length of fasting, method of blood draw, time of day for sampling, and method of quantification. Therefore, animals and samples should be manipulated and measured in a consistent way so that the results are readily comparable. In general, only small differences will be observed except under spectacular conditions. When abnormalities of lipid and glucose homeostasis are suspected, a second serum analysis after a shorter (4-hr) fast, or even in absence of fasting, is advisable. Furthermore, retesting in older mice yields quick information on the evolution of biochemical and metabolic parameters.

Manual determination of lipid parameters has a number of disadvantages, since reconstituted reagents are generally good for only 3 weeks. Therefore, samples are usually collected and then batch processed for efficiency. In addition, it is more difficult to take advantage of interlaboratory control systems, which are meant for high-throughput automated

machines. Use of automated systems allows for many more blood parameters to be analyzed simultaneously and requires a smaller volume of serum per test. This can leave sufficient sample for other measurements needed for a good first metabolic screen, including measurement of liver enzymes such as AST and ALT, urea, creatinine, total protein, albumin, glucose, insulin, and total bilirubin.

In general, plasma samples, which are collected in the presence of an anticoagulant, are preferred over serum samples, especially for the automated procedure, as the fibrin in serum can cause clogging of the tiny injection needles. In most cases, either EDTA or heparin can be used as the anticoagulant. In the case of FFA measurements, however, heparin should be avoided because it may activate lipoprotein lipase, which can result in false increases in FFA levels. The range of sensitivity for each enzymatic reaction as specified by the manufacturer should be respected; when samples are too lipemic, they need to be diluted in saline and a correction factor must be applied. Additional considerations for each kit are also given by the manufacturer and should be observed. For example, because the concentration of unesterified fatty acids rises quickly in due to the lipolysis of acylated lipids, FFA determination should be performed soon after the blood draw or on freshly frozen samples. Furthermore, exceeding the recommended incubation time for FFA reactions can lead to aberrantly increased values. This is in contrast to the cholesterol reaction, which is complete in about 2 min but shows no change in absorbance when incubation is continued for another 30 min. In general, these parameters should be carefully standardized so that intra-assay variation is minimal, a task that is more challenging with the manual method.

Lipoprotein analysis

Gel-exclusion chromatography is a quicker method than sequential ultracentrifugation for the separation of lipoproteins, but there is sometimes a disparity between the fractions isolated by centrifugation and by chromatography. This disparity is at least partially due to the difficulty in deciding where the boundaries between the conventional fractions lie on the chromatographic elution curve.

Measuring the protein content of each of the isolated fractions either by UV absorption or by a modified Lowry assay (Markwell et al., 1978) allows for the estimation of particle size. This is particularly relevant in the analysis of LDL because the protein measurement in the LDL fraction represents the quantity of apoB, as only one apoB exists per LDL particle. Thus, a higher amount of protein can reflect a greater number of particles. When cholesterol content is normalized to protein content, LDL particle density can be compared. As small dense LDL are associated with increased risk of CAD, this is a particularly important parameter (Lada and Rudel, 2004).

Tissue lipid content

Some optimization of this protocol may be required, depending on the mouse model and tissue type used. More lipids will accumulate in a high-fat-diet mouse model than in a chow-fed mouse model. Sample volumes and/or quantity of tissue used for extraction should be titrated such that the final reaction product is within the linear range of the system as stated by the manufacturer.

Bile acid measurements

Most of the bile acids of the enterohepatic circulation can be found in the gallbladder and the small intestine. The quantitative contribution of bile acids in the liver is relatively small. Therefore, it is not critical to extract the whole liver to determine bile acid pool size; however, it is critical to prevent any loss of bile from the gallbladder and contents from the small intestine, since this will affect the quantitative recovery of bile acids.

Several types of colorimetric assays are available for use in the measurement of total bile acids (see Sigma-Aldrich, Randox, BioQuant, DCL, bioMerieux). The general enzymatic principle is that the bile acids are enzymatically oxidized by the enzyme 3α-hydroxysteroid dehydrogenase, with the concurrent reduction of NAD^+ to NADH. However, in the fasting state and early stages of disease, levels of bile acids are low, and relatively few molecules of bile acid are available for the enzyme to act upon. Consequently, only a small amount of NAD^+ is reduced to NADH, making it analytically difficult to directly measure the change in NADH by absorbance at 340 nm. Assay sensitivity is increased by coupling NBT to NADH, since this produces a far greater change in absorbance (now measured at 540 nm) per molecule. Post-prandial lipemia and/or hemolysis interfere with this method (Mashige et al., 1978). In some instances, ultracentrifugation and overnight refrigeration of lipemic specimens can reduce the interference.

In addition to colorimetric assays, a radioimmunoassay (RIA) can also be used to

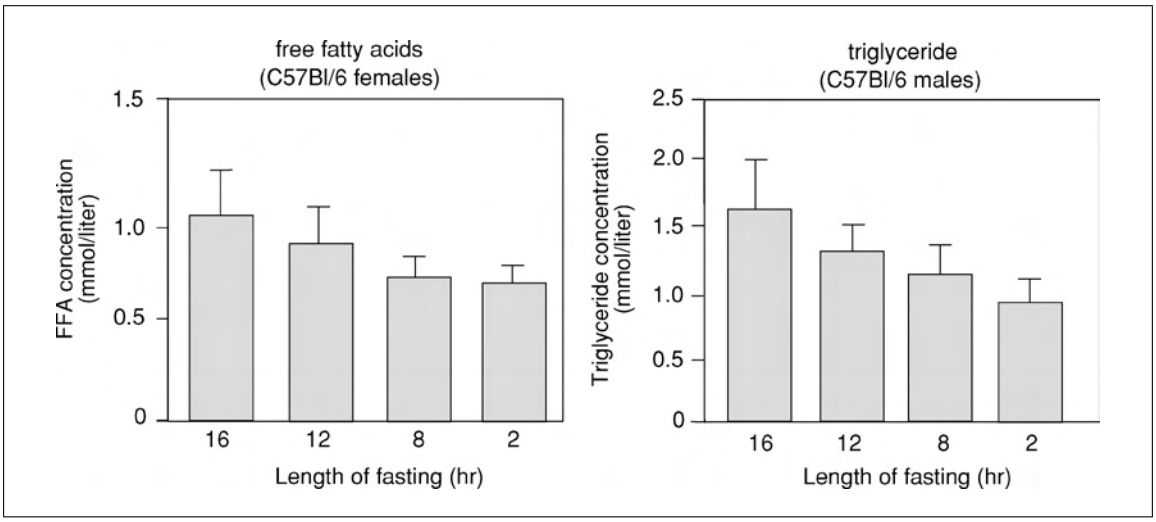
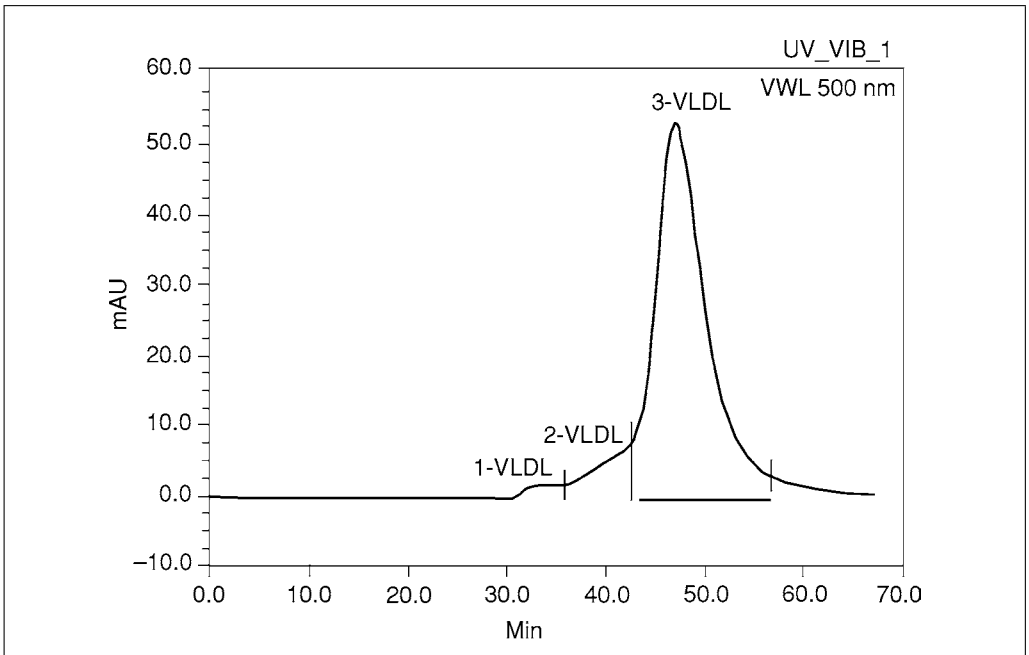


Figure 29B.2.5 Effect of length of fasting on FFA and TG plasma level in C57Bl/6 mice.



No.	Ret. time	Peak name	Height	Area	Rel. area	Amount	Type
	min		mAU	mAU * min	%		
1	33.24	VLDL	2.042	8.561	2.36	0.064	M *
2	38.94	LDL	4.488	31.282	8.61	0.233	M *
3	47.13	HDL	53.560	323.632	89.04	2.413	M *
Total			60.090	363.476	100.00	2.710	

Figure 29B.2.6 Typical lipoprotein profile in plasma of C57Bl/6 mice.

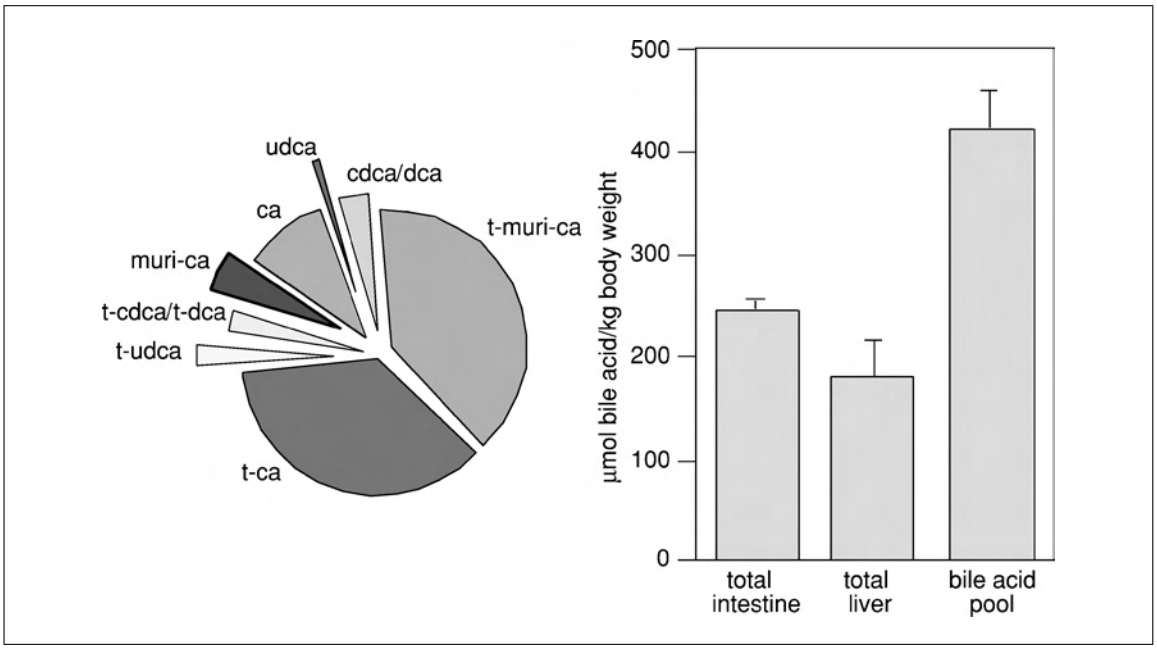


Figure 29B.2.7 A typical example of the composition and size of the bile acid pool. Abbreviations: ca, cholic acid; cdca, chenodeoxycholic acid; dca, deoxycholic acid; muri-ca, muricholic acid; udca, ursodeoxycholic acid; and t-, taurine conjugates.

measure total serum bile acid concentrations. An RIA (e.g., *UNIT 10.24*) uses a labeled iodine tracker and an antiserum against the amino acids (taurine or glycine) to which the bile acid is conjugated. Several differences have been noted between the results produced by these techniques, including different absolute values of bile acids, differential interference with hemolysis and lipemia, and differences in the volume of serum required. The authors suggest that careful consideration be given by the user to the appropriateness of the method, depending on individual needs.

Blood draw and handling

If analyses are not performed on the day of blood collection, samples should be stored at −20°C or −80°C. Tests should preferably be performed within 10 days of collection. Stability and storage depend on the different parameters to be determined. Plasma samples can be used undiluted, or diluted with deionized water if the volume is insufficient. In cases where the blood is hemolyzed, hemoglobin can sometimes interfere with certain measurements, and either a correction factor and/or resampling is suggested.

Anticipated Results

Blood lipid content

Blood lipid levels are generally reported in units of mM or mg/dl. The conversion factors for changing cholesterol, TG, and FFA concentration from mg/dl to mM are 2.586, 1.126, and 0.035, respectively. Blood lipid values are highly influenced by genetic background and environmental factors. Classically, C57BL/6 mice have low plasma cholesterol and low plasma triglyceride (TG) but high plasma glucose levels, whereas C3H mice have high plasma cholesterol, TG, and glucose levels, and 129 inbred strains have high plasma cholesterol and low TG levels. Environmental factors such as diet and length of fasting will also impact some lipid variables (see Fig. 29B.2.5).

Lipoprotein analysis

A typical lipoprotein profile as determined by FPLC is shown in Figure 29B.2.6. Lipoprotein analysis according to differential ultracentrifugation is represented as the amount of lipoprotein (cholesterol or TG) per µg of protein or is normalized to the initial volume of serum.

Tissue lipid content

TG, CE, and free cholesterol content of tissue lipid is generally expressed as mg lipid/mg tissue.

Bile acid measurements

An illustration of bile acid composition analysis is shown in Figure 29B.2.7. In mice, most of the bile acids are conjugated to taurine. Glycine conjugates are practically absent. In the liver and gallbladder, ∼95% of the bile

acids are conjugated, whereas in the intestine 30% of the bile acids are deconjugated by microbial enzymes. Typically 42% to 46% of the bile acids are present in the liver and gallbladder; the rest can be found in the small intestine. The hydrophobic-hydrophilic balance of the pool that results from the characteristics and proportions of the individual bile acids present within the pool is critical, as it may dictate most of the effects of bile acids on hepatic cholesterol metabolism (Mashige et al., 1978). For example, hydrophobic bile acids (e.g., CDCA) seem to increase biliary lipid secretion and inhibit cholesterol and bile acid synthesis, whereas hydrophilic bile acids (e.g., CA) are less effective.

In general, the protocol for testing bile acids entails blood sampling after a period of fasting followed by refeeding. Fasting levels of serum bile acids can be used for the diagnosis and prognosis of liver disease. Post-prandial bile acid measurements are also sensitive indicators of liver, intestinal, and gallbladder function. In this sense, bile acid measurements are considered to be a superior indicator of liver disease, compared to serum liver enzyme measures.

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Evaluation of Glucose Homeostasis

The development of glucose intolerance and insulin resistance (IR), characteristics of type 2 diabetes mellitus (T2DM), is a multistep process that is influenced by both genetic and environmental factors. As T2DM is currently the most common endocrine disorder in humans, the detailed understanding of these processes has been critically sought, with the mouse as the leading model. The ability to measure IR and glucose tolerance in the mouse is important both for understanding the pathogenesis of T2DM and for testing potential therapeutic interventions. When an initial blood screen in the mouse indicates altered levels of insulin and/or glucose, a number of well-established tests used to estimate glucose homeostasis and insulin sensitivity should be considered. These tests include model assessments, glucose/insulin infusion sensitivity tests, and clamps.

Because no single test is appropriate under all circumstances and there is considerable variation in the complexity and labor intensity of these various methods, care should be taken in prioritizing them. In general, the choice of method depends on the size and type of study to be undertaken, the goal of the experiment, and the technical expertise at hand. In this unit, tests for exploring glucose homeostasis and insulin sensitivity are described in order of technical invasiveness and ease, with the least invasive described first. In general, this is also the order in which the researcher should evaluate glucose homeostasis and insulin sensitivity in a mouse model, with the importance of each additional test being dependent on the result of the previous test. Thus, although the euglycemic clamp has been referred to as the gold standard for measuring IR, the relevance of performing this test must be considered if prior screening gives no indications that glucose homeostasis or IR is affected.

STRATEGIC PLANNING

The circumstances under which insulin sensitivity and/or glucose tolerance are measured highly influences their interpretation. In fact, insulin sensitivity changes not only in pathological situations but also during normal physiological stresses. To make any consistent assessment of overall insulin sensitivity, it is important to report any specific terminology or descriptors associated with the circumstances of measurement. More importantly, strategic planning is required to optimally control for the tissue-specific, metabolite-specific, and process-specific effects that can influence the conclusions drawn from an experiment. For example, when measuring the effect of IR by following the metabolic clearance of glucose, glucose-mediated glucose uptake must be taken into account, since it is greatly dependent on physical activity. Glucose is liberated from glycogen stores upon exercise, and the capacity to clear glucose from the circulation also increases. Stress and the time of day are also important determinants of IR. In general, mice have increased cortisol levels in the evening, leading to higher hepatic glucose output that can inflate IR. Similarly, increased adrenaline and cortisol levels due to stress can increase hepatic glucose output and consequently affect the assessment of IR. Furthermore, most methods used in the assessment of insulin sensitivity examine the insulin response of a single metabolite, glucose, primarily in the muscle and liver, and under fasting conditions. Because these results reflect only the conditions under which they were obtained, care should be taken not to over-interpret the data.

All these types of confounders and their potential influence on the ability to compare IR among various mouse models prompted the EU-funded Eumorphia program

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(*http://www.eumorphia.org*) to standardize several of the parameters associated with measures of glucose homeostasis and IR. The standardized conditions that are presented here as individual protocols are mostly based on the Eumorphia guidelines. However, individual investigators may need to modify these protocols according to their specific needs. A summary of the methods described here is presented in Table 29B.3.1.

NOTE: The protocols described here involve mouse techniques ranging from very basic (e.g., picking up a mouse) to very specialized (e.g., catheter surgery). All personnel taking part in these techniques must be trained beforehand to obtain results while minimizing stress-induced effects on the mouse. Protocols for these techniques can be found in detail elsewhere (Hedrich, 2004). All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Table 29B.3.1 Summary of Methods Used to Assess Glucose Tolerance and Insulin Sensitivity[a]

Test	Labor intensity	Method	Interpretation
FPG and FPI	One blood sample (50–100 μl; tail bleed or retro-orbital)	Overnight or 4 hr fasting glucose and insulin measurements	Estimate of basal IR
HOMA	Simple calculations	Two formulas using FPG and FPI	Estimate of basal IR and β–cell function (not validated in mouse)
MTT	Two samples (50–100 μl; tail bleed or retro-orbital)	Overnight fast, and glucose and insulin measurements ± a standardized meal	Estimate of basal and postprandial IR; intestinal nutrient absorption taken into account
OGTT	Eight small samples (∼3 μl) over 180 min (tail bleed)	Overnight fast, and glucose measurements after oral glucose load	Estimate of IR based on the evolution of glucose levels; intestinal glucose absorption taken into account
IPIST	Six small samples (∼3 μl) over 90 min (tail bleed)	Overnight fast, and glucose measurements after IP insulin load	Estimate of basal insulin sensitivity based on the evolution of glucose levels
IPGTT	Eight small samples (∼3 μl) over 180 min (tail bleed)	Overnight fast, and glucose measurements after IP glucose load	Estimate of IR based on the evolution of glucose levels
Euglycemic clamp	Catheter surgery, small blood samples every 5 to 10 min over 180 min (tail bleed)	Constant rate insulin infusion, variable rate glucose infusion; frequent glucose measurements	Estimate of IR based on average glucose infusion rate over the last 60 min; often regarded as the gold standard

[a]FPG, fasting plasma glucose; FPI, fasting plasma insulin; IR, insulin resistance; HOMA, homeostatic model assessment; MTT, meal tolerance test; OGTT, oral glucose tolerance test; IPIST, intraperitoneal insulin sensitivity test; IPGTT, intraperitoneal glucose tolerance test.

GLUCOSE MEASUREMENT BY BLOOD GLUCOSE MONITOR

Circulating glucose is the first and foremost biochemical measure to be taken when evaluating the metabolic phenotype of mice, as changes in this vital and typically strictly regulated metabolite invariably indicate alterations in some aspect of metabolism.

The limited blood volume of the mouse imposes special requirements for the sensitivity of the technique by which any circulating metabolite or substance, including glucose, is measured. In the case of glucose, the problem is exaggerated by the fact that several of the metabolic test protocols provided in this unit require repeated glucose measurements from the same mouse over relatively short intervals. In addition, some tests (e.g., euglycemic clamp) require virtually on-line information about blood glucose levels, setting strict limits for the rapidity of the glucose measurement. Within these parameters, the best method for measuring blood glucose during mouse metabolic phenotyping experiments is usually the blood glucose monitor designed for diabetic humans. These monitors are readily available, easy to use, and relatively inexpensive. Most importantly, they provide reliable results within seconds from a small sample of blood.

This protocol outlines the use of the Accu-Chek Active blood glucose monitor from Roche Diagnostics. For specifics and troubleshooting, refer to the manufacturer's user manual. Examples of similar products include OneTouch Ultra (LifeScan), BD Logic Blood Glucose Monitor (BD), Precision Xtra (Abbott), and HemoCue Glucose (HemoCue). Other techniques for measuring blood glucose, more suited for the analysis of frozen plasma or serum samples at some later point in time, include enzymatic methods, mostly utilizing either hexokinase with glucose-6-phosphate dehydrogenase or glucose oxidase. These methods can be performed manually (e.g., Glucose (HK) assay kit; Sigma) or automatically (e.g., AU-400 automated laboratory work station with OSR6121 glucose reagent; Olympus).

Materials

Mice

Low- and high-level glucose control solutions (e.g., Accu-Chek active glucose control solutions, Roche Diagnostics; low concentration: 50 mg/dl, reference range 42 to 72 mg/dl, and high concentration: 300 mg/dl, reference range 290 to 328 mg/dl)

Blood glucose monitor and associated glucose test strips for glucose measurement (e.g., Accu-Chek Active, Roche Diagnostics)

Scalpel blade

1. Calibrate the blood glucose monitor by first inserting the code key into the slot on the left side with the monitor powered off. Then insert a test strip and verify that the code appearing on the monitor matches that on the test strip vial.

   Perform this step every time a new box of test strips is opened.

2. Perform a performance check on the monitor. Insert a fresh test strip, and after the monitor is activated, press the button marked S. Carefully dispense one drop from the low-level control solution bottle and touch it to the center of the orange pad on the strip. Verify that the result, appearing in a few seconds, is within the low range indicated on the test strip vial. In cases where high glucose values are expected, repeat this procedure using a high-level control solution.

   A performance check should be done every time a new box of test strips is opened, or if the performance of the monitor is in doubt. It is best to use the same lot number of strips for one experiment, as lot-to-lot variations can occur.

3. Insert a fresh test strip.

   The blood sample must be applied within the next 2 min before the monitor powers off.

4. Draw a small (~3 μl) blood sample from a mouse by nicking the lateral tail vein 1 to 2 cm from the tip with a scalpel blade (see *UNIT 29A.3*).
5. Transfer the drop of blood to the orange pad on the test strip, preferably by touching the strip directly to the bleeding tail wound, or by pipeting. Record the result, which appears in 5 sec.
6. Apply direct pressure to the incision until the blood has clotted and return the mouse to its cage.

BASIC PROTOCOL 2

INSULIN MEASUREMENT BY ELISA

In addition to glucose, levels of circulating insulin are often of interest due to the importance of this β-cell hormone on the regulation of circulating glucose levels. In general, changes in insulin appear earlier than changes in glucose during the progression of experimental metabolic interventions.

This protocol outlines the use of the Ultrasensitive Mouse Insulin ELISA from Mercodia. This solid-phase direct sandwich ELISA uses two monoclonal antibodies raised against separate antigenic sites on the insulin molecule (for additional description of sandwich ELISAs, see *UNIT 11.2*). Insulin in the sample is immobilized by one anti-insulin antibody bound to the assay well, and is labeled by a second anti-insulin antibody conjugated with peroxidase. Unbound enzyme-conjugated antibody is removed during washing, and the bound conjugate is detected colorimetrically. For additional information, refer to the manufacturer's instructions. Other sources for sensitive ELISA kits for measuring insulin in small volumes of mouse blood or other samples include Alpco Diagnostics, Crystal Chem, and Linco Research.

CAUTION: The stop solution in this kit contains 0.5 M H_2SO_4. Follow routine precautions for handling hazardous chemicals.

Materials

Ultrasensitive Mouse Insulin ELISA kit (e.g., Mercodia) containing:
  96-well microtiter plates coated with mouse monoclonal anti-insulin antibody
  Calibrators (i.e., standards)
  Antibody-enzyme conjugate (concentrate) and buffer
  Wash buffer (concentrate)
  TMB substrate reagent (3′,3′,5′,5′-tetramethylbenzidine; light sensitive)
  Stop solution: 0.5 M H_2SO_4
Mouse serum or plasma
0.9% (w/v) NaCl

Microtiter plate shaker
Microtiter plate washer (optional)
Plate reader capable of measuring at 450 nm

1. Pipet 25 μl of calibrator 0 into each well of a 96-well microtiter plate coated with mouse monoclonal anti-insulin antibody.
2. Pipet calibrators (5 μl) and mouse serum or plasma (5 μl) into duplicate wells of the antibody-coated microtiter plate.
3. Add 50 μl of antibody-enzyme conjugate reagent to each well.

   Prepare by diluting the enzyme conjugate concentrate eleven-fold with enzyme conjugate buffer, e.g., add 600 μl to 6 ml of buffer per one 96-well assay. Store up to 2 months at 2° to 8°C.

4. Incubate on a shaker for 2 hr at room temperature (18° to 25°C).

5. Aspirate the reaction volume, add 350 µl of wash buffer to each well, and aspirate completely. Repeat five times for a total of six washes.

   Prepare the wash buffer by diluting the wash buffer concentrate 21-fold with redistilled water, e.g., add 35 ml to 700 ml of redistilled water per one 96-well assay. Store up to 4 weeks at 2° to 8°C.

   Washing can also be performed using an automated microtiter plate washer.

6. After the final wash, invert the plate and tap firmly against absorbent paper.
7. Add 200 µl TMB substrate reagent to each well.
8. Incubate 30 min at room temperature.
9. Add 50 µl stop solution.
10. Place the plate on the shaker for ~5 sec to ensure mixing of substrate and stop reagents.
11. Measure the absorbance at 450 nm and calculate the results according to the manufacturer's manual.

    In short, for computerized analysis, plot the absorbance values (y-axis) for the calibrators against the corresponding insulin concentrations (x-axis) as a log-log graph, and fit a cubic spline regression curve (also called third-order polynomial trend line) to obtain the insulin concentrations for the samples. Sometimes a fourth-order polynomial trend line can provide more accurate results. Alternatively, plot the absorbance values on the x-axis against the insulin concentrations on the y-axis, and fit a power trend line to obtain an equation that is easier to solve than above. For manual calculation, plot the calibrators on a log-log or log-lin paper as above, and read the insulin concentrations for each sample from the curve.

12. If the insulin concentration exceeds the value of the highest calibrator, dilute the sample ten-fold with 0.9% NaCl and repeat the analysis.

HOMEOSTATIC MODEL ASSESSMENT

BASIC PROTOCOL 3

The homeostatic model assessment (HOMA) is a mathematical model that takes into account several parameters known to effect glucose homeostasis. It relies on experimental human data, and allows for values of insulin sensitivity and β-cell function to be obtained when simultaneous fasting plasma glucose and fasting insulin concentrations are known (Matthews et al., 1985; Levy et al., 1998; Wallace et al., 2004). The solutions for the full model have highly significant correlation to euglycemic clamp results in humans (Wallace et al., 2004). Due to their nonlinear nature, they are best calculated using a computerized program available at *http://www.dtu.ox.ac.uk/*. Two simple formulas can be used to approximate the HOMA indices, one for insulin resistance (HOMA-IR) and one for β-cell function (HOMA-%B). This protocol describes these two simple formulas. It should be noted that the validation of HOMA on mice is lacking, so these indices should be used with caution until such validation becomes available.

Materials

Mice
Heparinized tubes for blood sampling (e.g., Microvette, Sarstedt)

Mouse cages
Scalpel blade
Refrigerated microcentrifuge

Additional reagents and equipment for measuring blood glucose (see Basic Protocol 1) and insulin (see Basic Protocol 2)

1. Fast mice 14 to 16 hr (overnight). Ensure that they have ad libitum access to drinking water.
2. Obtain ~100-µl blood samples in heparinized tubes by nicking the lateral tail vein 1 to 2 cm from the tip with a scalpel blade (see *UNIT 29A.3*). Apply direct pressure to the incision until the blood clots and then return the mouse to its cage.

   A blood glucose measurement can also be taken from whole blood before plasma separation, using a blood glucose monitor (see Basic Protocol 1).
3. Microcentrifuge the blood samples 15 min at 2300 × *g* (5000 rpm), 4°C. Collect the plasma (~40 µl) into clean tubes, and store at −20° or −80°C until glucose and insulin measurement (up to 1 month for glucose and 6 months for insulin).
4. Measure blood glucose (see Basic Protocol 1) and insulin (see Basic Protocol 2) levels.
5. Calculate the HOMA-IR and HOMA-%B results according to the following two equations:

$$\text{HOMA-IR} = \frac{\text{FPI} \times \text{FPG}}{22.5 \times 18}$$

$$\text{HOMA-\%B} = \frac{(20 \times \text{FPI})}{\frac{\text{FPG}}{18} - 3.5}$$

   where FPI is the fasting plasma insulin concentration (µU/ml) and FPG is the fasting plasma glucose concentration (mg/dl).

   Glucose values with mmol/liter as a unit can also be used by removing the factor 18 from the formulas (for glucose, 1 mM = 18 mg/dl). Insulin values with pmol/liter as a unit can be converted to µIU/ml by dividing values by 6.945, or from µg/ml to IU/ml by dividing values by 45.5 (for insulin, 1 µU/ml = 6.945 pmol/liter and 1 IU = 45.5 µg of standard preparation).

BASIC PROTOCOL 4

MEAL TOLERANCE TEST

The meal tolerance test (MTT) is a simple method to explore glucose metabolism and insulin secretion following a meal by measuring fasting and fed glucose and insulin levels. First, animals fast for 14 to 16 hr, after which a fasting blood sample is collected by retroorbital venipuncture (Hedrich, 2004). The same animals are then fasted again 1 week later, and a calibrated meal (normally chow diet) is provided for 1 hr in the form of a specific weighed amount of food pellets. Alternatively, a standardized complex nutrient solution (e.g., Ensure Plus, Abbott Nutrition) can be administered in the beginning of the feeding period by oral gavage. A post-prandial blood sample is collected immediately after the 1-hr feeding period.

An alternative to collecting blood on separate days for the fasting and fed states is to use, instead of retroorbital venipuncture under anesthesia, blood sampling from the tail vein (*UNIT 29A.3*) before (fasting state) and after (fed state) the feeding period (see Basic Protocol 3). However, since stress may alter the feeding behavior of mice, this alternative is mostly useful with the administration of nutrient solution rather than with free pellet feeding.

Materials

Mice
Isoflurane inhalation anesthesia unit (composed of a mixing and flow-control system for O_2/isoflurane anesthesia; e.g., TEM, *http://www.TEM.fr*)
Non-heparinized glass capillaries, 50- to 100-μl (e.g., Vitrex by Modulohm)
Mouse chow

Mouse cages
Refrigerated microcentrifuge

Additional reagents and equipment for measuring blood glucose (see Basic Protocol 1) and insulin (see Basic Protocol 2) levels

1. Fast mice for 14 to 16 hr (overnight). Ensure that they have access to drinking water at all times.
2. On the following day, before 8 a.m., prepare experiment record sheet, tubes for blood collection, and strips for glucose measurement. Calibrate the blood glucose monitor.
3. At 8 a.m., anesthetize a mouse lightly by isoflurane (see *UNIT 29B.1*, Basic Protocol 1) and take a 50- to 100-μl blood sample by retroorbital venipuncture (see Hedrich, 2004) into a non-heparinized capillary. Drop a small amount (~3 μl) of blood onto a test strip placed in the blood glucose monitor (see Basic Protocol 1), and collect the rest of the 50- to 100-μl sample in a non-heparinized capillary on ice. Record the glucose measurement from the monitor.
4. Microcentrifuge the blood samples 15 min at 2300 × *g* (5000 rpm), 4°C. Collect the plasma (~20 to 40 μl) into clean tubes, and store at −20° or −80°C until glucose and insulin measurement (up to 1 month for glucose and 6 months for insulin).
5. Repeat the fasting 1 week later. On the following day, at 8 a.m., place mice individually in fresh cages with water and a preweighed meal (approximately three pellets of chow diet) for 60 min.
6. Prepare for blood sampling and glucose measurement as in step 2.
7. After the 60-min meal, weigh the remaining food.
8. Take blood samples as described in steps 3 and 4.
9. Perform glucose and insulin measurements according to Basic Protocols 1 and 2.
10. For data analysis, divide the increase in glucose and insulin values over fasting levels for each mouse by the weight of consumed food in grams.

    Thus, results are reported as mg/dl•g^{-1} for glucose and ng/ml•g^{-1} for insulin. Sometimes, it is also useful to express the results as per calories consumed.

ORAL GLUCOSE TOLERANCE TEST

BASIC PROTOCOL 5

The oral glucose tolerance test (OGTT) measures the clearance of a standardized glucose load from the body. Since the glucose bolus enters the body via the natural route—it is absorbed from the intestinal tract—this test also takes into account intestinal aspects of glucose absorption. Animals undergo fasting for ~14 to 16 hr, a glucose solution is administered by oral gavage (Hedrich, 2004), and blood glucose is measured at different time points in a period of 3 hr.

An important consideration for this procedure is the size of the cohort to be assessed at any one time. For two trained persons, a maximum of sixteen animals is recommended. To minimize variation due to technical aspects, each person should be designated a role in the experiment, i.e., only one person/experiment gavages all mice. Therefore, careful planning is needed when dividing an experimental cohort of more than sixteen of animals.

Materials

Mice
20% (w/v) aqueous glucose solution (see recipe), sterile

Mouse cages
Animal scale
Blood glucose monitor and associated glucose test strips for glucose measurement (e.g., Accu-Chek Active, Roche Diagnostics)
1-ml syringes (e.g., Terumo) and 22-G ball-tip needle (e.g., Popper and Sons)
Scalpel blade
Timer

1. Fast mice for 14 to 16 hr (overnight). Ensure that they have access to drinking water at all times.
2. On the following day, at 8 a.m., place mice individually in fresh cages with only water (no food). Identify cages with the mouse number.
3. Record the weight of each mouse to within 0.1 g.
4. Calculate and record the volume of 20% glucose solution required for oral gavage of 2 g glucose/kg at an oral injection volume of 10 μl/g body weight.

   For a mouse with a body weight of 30 g, inject 300 μl of glucose solution.

5. Prepare experiment record sheet, strips for glucose measurement, and a 1-ml syringe for each animal containing the calculated volume of glucose to be gavaged.

   Unlike for intraperitoneal tests (see Basic Protocols 6 and 7), it is practical and more economic to use just one ball-tip needle, which is switched from syringe to syringe as needed, with brief external rinsing in 70% ethanol and distilled water between uses.

6. Calibrate the blood glucose monitor with the standard strip.
7. Measure the basal glucose concentration ($T = 0$) in each mouse by removing one mouse at a time from its cage, placing it on the top of its cage, and making a small incision over the lateral tail vein (1 to 2 cm from the tail tip) using a scalpel blade (see *UNIT 29A.3*). Touch a small (~3 μl) blood sample directly onto the test strip placed in the blood glucose monitor (see Basic Protocol 1). Apply direct pressure to the incision until the blood clots and return the mouse to its cage.
8. After all mice have been measured for basal glucose concentrations, administer the glucose solution by oral gavage (Hedrich, 2004) to each animal at a 30- or 60-sec interval between animals, depending on the size of the experimental cohort. Start the timer with the first mouse gavaged.

   In general, it takes a minimum of 30 sec for an experienced person to perform the gavage and/or take a blood measurement. All injections must be finished within 15 min in order to perform the next blood glucose measurement at T = 15 min.

9. At $T = 15$ min, measure the blood glucose again, starting with the first mouse gavaged and using the same time interval used for gavage, until all the mice in the

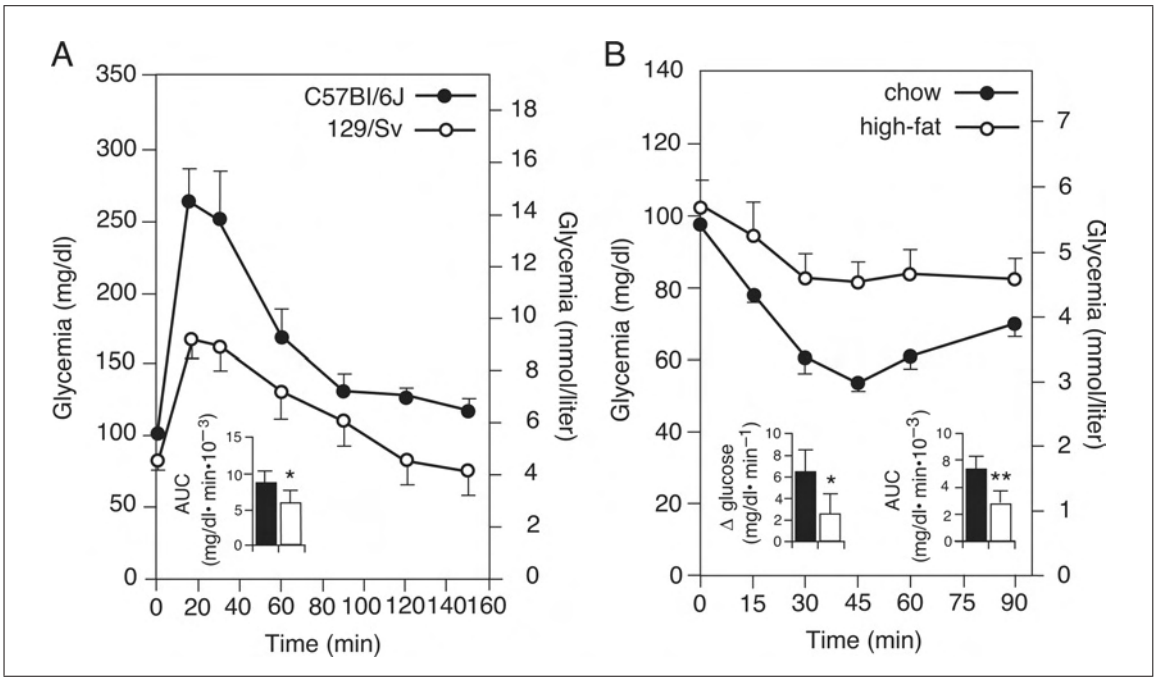

Figure 29B.3.1 Examples of the intraperitoneal insulin sensitivity test (IPIST) and intraperitoneal glucose tolerance test (IPGTT). Note that the figures represent different sets of mice. (**A**) An IPGTT showing a difference in glucose tolerance between wild-type C57Bl/6J and 129/Sv mice, the former being more insulin resistant. (**B**) An IPIST on chow-fed and high-fat-fed mice, showing decreased insulin sensitivity in the high-fat-fed group.

cohort have been measured. It is important that timing be followed as closely as possible. To restart the bleeding, remove the clot from the first incision and massage the tail if blood flow is inadequate.

10. Repeat at $T = 30$, 60, 90, 120, 150, and 180 min after gavage.

11. At the end of the experiment, return the mice to their original cages and provide plenty of food and water. Ensure that no animal is bleeding excessively.

12. For visual representation, plot glucose values versus time (see Fig. 29B.3.1A for an example of a graph from on IPGTT).

13. For statistical analysis, calculate the area under the curve (AUC) for each mouse using the equation:

$$\mathrm{AUC} = \sum_{n=1}^{x-1}\left(\left(T_{n+1} - T_n\right) \times \left(\frac{\left|\mathrm{Glc}_{T_n} - \mathrm{Glc}_{T_{n+1}}\right|}{2} + \min\left(\mathrm{Glc}_{T_n}, \mathrm{Glc}_{T_{n+1}}\right) - \mathrm{Glc}_{T_0}\right)\right)$$

where Glc is glucose concentration, T is time, n is the time point number, x is the total number of time points, and $\min(\mathrm{Glc}_{Tn}, \mathrm{Glc}_{Tn+1})$ is the lower of two consecutive glucose concentrations.

The rationale for this calculation is depicted in Figure 29B.3.2.

To exclude the effect of differences in fasting glucose levels between animals, AUC is usually calculated as incremental area ('above baseline'), where only the area above the fasting level for each mouse is taken into account.

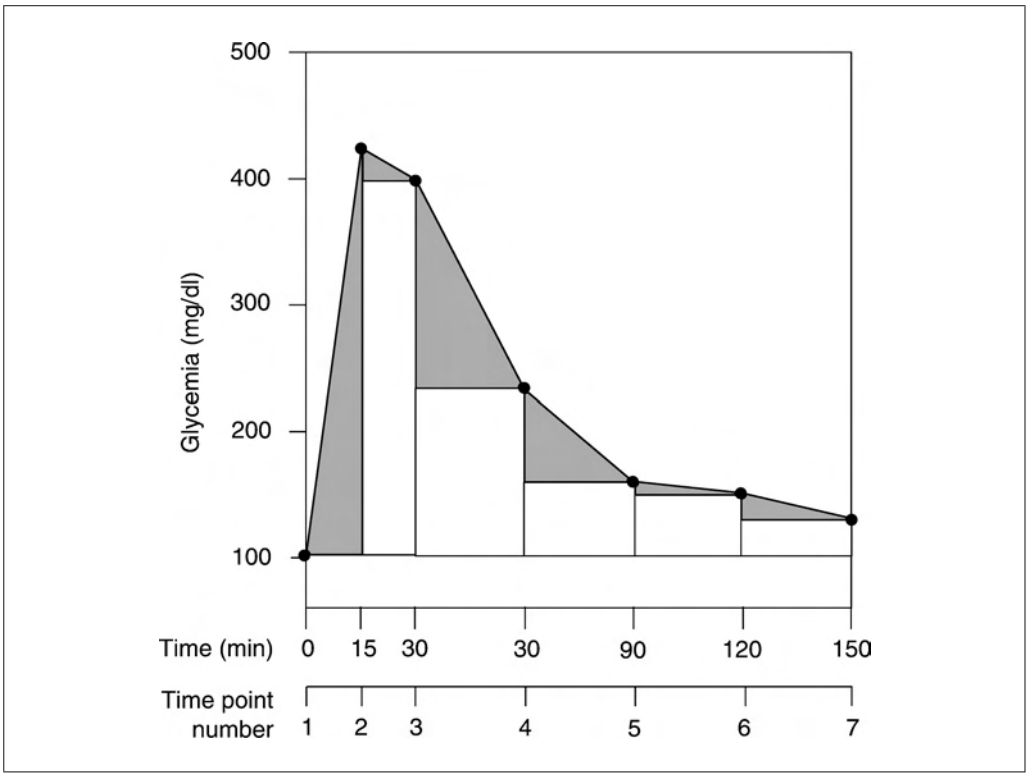

Figure 29B.3.2 Rationale for calculating the area under the curve (AUC). Areas of the triangles (gray), delimited by two consecutive time points and glucose values therein, and quadrangles (white), below each triangle but above baseline value, are summed.

BASIC PROTOCOL 6

INTRAPERITONEAL INSULIN SENSITIVITY TEST

The intraperitoneal insulin sensitivity test (IPIST) measures glucose levels subsequent to a standardized insulin load. It gives an estimate of the insulin sensitivity of the animals. Animals are fasted for 14 to 16 hr, a bolus of insulin is administered intraperitoneally (i.p.), and blood glucose is measured at different time points during 90 min.

An important consideration for this procedure is the size of cohort to be assessed at any one time. For two trained persons, a maximum of sixteen animals is recommended. To minimize variation due to technical aspects, each person should be designated a role in the experiment, i.e., only one person/experiment administers insulin to all mice. Therefore, careful planning is needed when dividing an experimental cohort of more than sixteen animals.

Materials

Mice
Insulin solution for IPIST (see recipe)

Mouse cages
Animal scale
1-ml syringes (e.g., Terumo) and 25-G × 5/8-in. needles (e.g., Terumo)
Blood glucose monitor and associated glucose test strips for glucose measurement (e.g., Accu-Chek Active, Roche Diagnostics)
Scalpel blade
Timer

1. Fast mice for 14 to 16 hr (overnight). Ensure that the animals have access to drinking water at all times.

2. On the following day, at 8 a.m., place mice individually in fresh cages with only water (no food). Identify cages with the mouse number.
3. Record the weight of each mouse to within 0.1 g.
4. Calculate and record the volume of insulin working solution required for an i.p. injection of 0.5 IU/kg in an injection volume of 3.6 μl/g body weight.

   For a mouse with a body weight of 30 g, inject 108 μl of insulin working solution.

5. Prepare the 1-ml insulin syringes and 25-G × 5/8-in. needles with the calculated volumes for each animal in the experiment. Prepare experiment record sheet and test strips for glucose measurement.
6. Calibrate the blood glucose monitor with the standard strip.
7. Measure the basal glucose concentration ($T = 0$) in each mouse by removing one mouse at a time from its cage, placing it on the top of its cage, and making a small incision over the lateral tail vein (1 to 2 cm from the tail tip) using a scalpel blade (see *UNIT 29A.3*). Touch a small (~3 μl) blood sample directly onto the test strip placed in the blood glucose monitor (see Basic Protocol 1). Apply direct pressure to the incision until the blood clots and return the mouse to its cage.
8. After all mice have been measured for basal glucose concentrations, administer the insulin solution by i.p. injection to each animal at a 30- or 60-sec interval between animals, depending on the size of the experimental cohort. Start the timer with the first mouse injected.

   In general, it takes a minimum of 30 sec for an experienced person to perform the injection and/or take a blood measurement. All injections must be finished within 15 min in order to perform the next blood glucose measurement at T = 15 min.

9. At $T = 15$ min, measure the blood glucose again, starting with the first mouse injected and using the same time interval used for injections, until all the mice in the cohort have been measured. It is important that timing be followed as closely as possible. To restart the bleeding, remove the clot from the first incision and massage the tail if blood flow is inadequate.
10. Repeat at $T = 30$, 60, and 90 min after insulin injection.

    CAUTION: *If at any time the blood glucose falls below ~36 mg/dl, the value should be confirmed. If the value is accurate, the mouse should be rescued by an injection of 0.5 to 1.0 g glucose/kg from a 20% aqueous glucose solution (see Basic Protocol 5), as very low values can be lethal to the animal.*

11. At the end of the experiment, return the mice to their original cages and provide plenty of food and water. Ensure that no animal is bleeding excessively.
12. For visual representation, plot absolute glucose values versus time (Fig. 29B.3.1B).

    In cases where groups differ strongly in FPG values, plotting the percent of fasting value versus time can be a viable alternative.

13. For statistical analysis, calculate the rate of decline in glucose between T_0 and T_{15} using log-transformed absolute glucose values. Also, calculate the inverse area above the curve (AUC_i) below baseline, again using absolute values, for each mouse as follows:

$$AUC_i = \sum_{n=1}^{x-1}\left(\left(T_{n+1} - T_n\right) \times \left(\frac{\left|Glc_{T_n} - Glc_{T_{n+1}}\right|}{2} + Glc_{T_0} - \max\left(Glc_{T_n}, Glc_{T_{n+1}}\right)\right)\right)$$

where Glc is glucose concentration, T is time, n is the time point number, x is the total number of time points, and $\max(Glc_{Tn}, Glc_{Tn+1})$ is the higher of two consecutive glucose concentrations.

In some cases, it may be useful to calculate AUC_i separately for the last 85 or 60 min of the test since, after the initial direct insulin effect, counter-regulatory hormones and gluconeogenesis will start playing a role. Thus, excluding the initial phase may provide additional phenotypic information.

BASIC PROTOCOL 7

INTRAPERITONEAL GLUCOSE TOLERANCE TEST

The intraperitoneal glucose tolerance test (IPGTT) measures the clearance of an intraperitonally injected glucose load into peripheral tissues. Compared to the oral glucose tolerance test (OGTT; see Basic Protocol 5), the IPGTT does not address mechanisms participating in glucose absorption from the intestine and hence can be considered a more "artificial" test than the OGTT. This procedure is performed exactly as the OGTT, except that the 20% aqueous glucose solution is administered by i.p. injection using a 25-G × 5/8-in. needle (e.g., Terumo) and the syringes, containing the calculated volume of glucose, should be prepared for each animal with the needles already attached and voided of dead volume. Similarly to oral administration, a 2 g glucose/kg dose is used, with an i.p. injection volume of 10 μl/g body weight. Considerations for careful timing and cohort size apply here as well.

BASIC PROTOCOL 8

HYPERINSULINEMIC-EUGLYCEMIC CLAMP

The current gold standard method for measuring IR is the euglycemic clamp, described in this protocol. In this method, glucose is "clamped" at a predetermined value (100 mg/dl or 5.5 mmol/liter for euglycemia) by titrating a variable rate of glucose infusion (glucose infusion rate or GIR) against a fixed rate of insulin infusion (resulting in hyperinsulinemia). Mice are permanently catheterized using a bifurcated catheter assembly to allow for simultaneous constant and variable infusion of insulin and glucose, respectively. The clamps are performed in awake, unrestrained, unstressed, and light-cycle-inverted mice following a 5-hr fast. Mice are acclimatized for at least 30 min to the tops of cages while their catheters are attached to a syringe-infusion pump. Baseline glucose values are measured by tail vein sampling prior to the injection of insulin. Catheter placement is assessed with a short priming dose (6 μl/min, 1 min) of insulin prior to the constant infusion of insulin at a flow rate of 2 μl/min equivalent to 18 mU of insulin/kg/min. Blood glucose values are monitored every 5 to 10 min throughout the test. Within the first 15 min, a decrease in blood glucose should be observed, at which point a 20% glucose (in saline) infusion is started. The GIR is varied as necessary until euglycemia (±15%) has been reached and maintained. The animal is "clamped" at this level, and the degree of IR is inversely related to the amount of glucose necessary to maintain the required blood glucose concentrations. The GIR at clamp (mg glucose/kg/min) is calculated as an average during the last 60 min of the experiment. When the average GIR of one animal is greater than another, it indicates better insulin sensitivity or that the clearance of glucose from the plasma is much faster.

Modifications to several parameters of this test—including the duration of fasting, the amount of insulin given at the initial infusion phase, and the inclusion of tracers—may be necessary, depending on the specific aspect of glucose homeostasis that is being studied. A validation of some of these parameters was recently summarized (Ayala et al., 2006).

The details of this procedure are based on assaying six animals at a time by two experienced experimenters. The number of mice will vary depending on the number of people

involved and the size of the experimental cohort required to obtain statistically relevant results. In general, a minimum of six animals per experimental group is required.

NOTE: Catheter surgery should be performed by an experienced veterinarian or other mouse care personnel competent in performing this procedure.

Materials

Mice
Anesthesia: ketamine and xylazine
Insulin solution for euglycemic clamp (see recipe)
20% (w/v) glucose in saline solution (see recipe), sterile
Saline: 0.9% (w/v) NaCl
70% (v/v) ethanol

Mouse femoral catheter (e.g., M-FAC, Braintree Scientific)
Mouse cages with flat-grated lid
Animal scale
1- and 2-ml syringes
Single syringe infusion pumps capable of holding a 2-ml syringe with a range of infusion rates of 5 to 20 μl/min (e.g., Harvard Apparatus or TSE Systems)
Multi-syringe infusion pump capable of holding a minimum of six individual 1-ml syringes with a range of infusion rates of 3 to 10 μl/min (e.g., Harvard Apparatus or TSE Systems)
Tubing (e.g., Tygon tubing, Ø 0.51 mm, Biorad)
Tubing connectors (e.g., 23-G needles, Terumo)
Y-shaped canula connector (e.g., C312ICP/O, Protech International)
Retort stand with a movable arm to hold the Y-shaped canula connector and tubing in place
Timer
Blood glucose monitors and associated glucose test strips for glucose measurement (e.g., Accu-Chek Active, Roche Diagnostics)
Scalpel blade

Prepare clamp set-up

Prior to the catheter surgery and clamp, mice should be acclimatized to a light-inverted cycle for at least 1 week.

1. At least 2 to 3 days before the experiment, place a catheter in the femoral vein of all mice (under anesthesia using ketamine and xylazine), with the catheter fed underneath the skin and affixed behind the head.

   The catheter should not require flushing prior to the experiment if it has been filled with saline and closed the day of the surgery.

2. After surgery, house mice individually and allow to recover for at least 48 hr, preferably long enough for them to regain their pre-operative body weight.

3. On the day of the clamp, remove the food from the mice 5 hr prior to the assay. Maintain access to water.

4. A minimum of 30 min prior to beginning the clamp, weigh the mice to within 0.1 g and place them on top of their cages to acclimatize.

5. Prepare the insulin working solution and fill six 1-ml syringes. Fill six 2-ml syringes with 20% glucose solution.

6. Place all infusion pumps on a shelf above the mice on their cages and insert the six glucose syringes vertically in the six single-infusion pumps. Attach syringes with appropriate tubing and connectors and make the connections from the pump to the

Y-shaped canula connector on the retort stand. Do not make the connection to the animal at this point. Start glucose infusion pumps at a high rate of infusion to fill the line with glucose and look for bubbles or plugs. Ensure that glucose is coming out of the end of the line at a rate comparable to the setting on the infusion pump. Ensure that the mice are not exposed to drips of glucose during the priming of the system.

7. While maintaining the glucose connections, place the six insulin syringes vertically in the multi-syringe infusion pump and connect the insulin syringes with tubing to the Y-shaped canula connector. Again, purge the lines with insulin by turning the infusion pumps on at a high rate. Ensure that insulin fills the lines and there are no bubbles or plugs.

   For the general setup, see Figure 29B.3.3.

8. Stop all infusion pumps. Reset the glucose pumps to 5 μl/min and the insulin pump to 2 μl/min.

   A flow rate of 2 μl/min is equivalent to 18 mU of insulin/kg/min based on an average mouse mass of 20 g. This amount of insulin is enough to obtain hyperinsulinemic conditions in a mouse. In general, this infusion rate of insulin is used even if the mass of each mouse is different so that only one pump is required (the validity of this approach is open for discussion).

9. Connect the infusion tubes to the mice. Ensure that the tubing is not twisted during the experiment and the mice are free to roam the top of the cage.

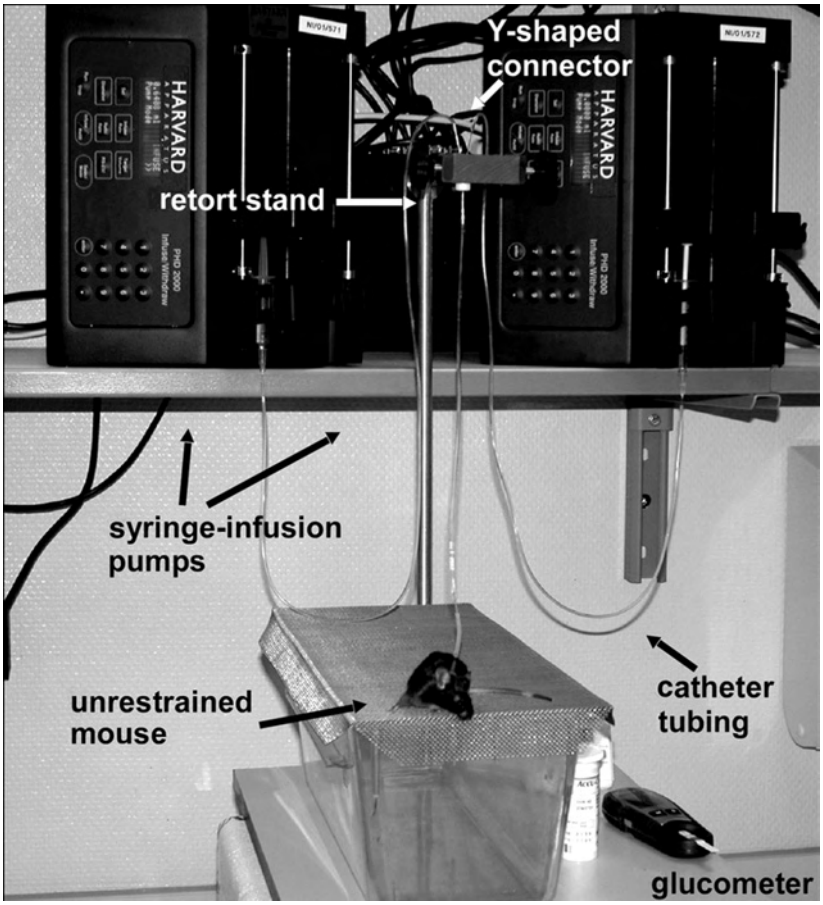

Figure 29B.3.3 An example of a set-up for a euglycemic clamp showing a single catheterized mouse.

Perform infusions and blood sampling

10. After 30 min of acclimatization, cut the tip of the mouse tail with a scalpel blade. Measure a small sample of blood with the blood glucose monitor to obtain the basal ($T = 0$) glucose value.

    This value should be near euglycemia or 100 mg/dl (5.5 mmol/liter).

11. Begin the timer and the insulin infusion pump, starting at an infusion rate of 2 μl/min. After 3 min, increase the insulin infusion rate to 6 μl/min and maintain for 1 min, and then return to 2 μl/min for the remaining clamp (180 min). Measure the plasma glucose value at 5-min intervals.

    The glucose should be lower at ~15 min than at time 0.

12. When the glucose is around euglycemia, begin the glucose infusion pumps, starting at an infusion rate of 5 μl/min.

    It is not necessary for all animals to start glucose infusion at the same time.

13. Continue to monitor plasma glucose every 5 min and increase or decrease the individual glucose infusion rates until euglycemia has been reached and maintained. Adjust glucose infusion rates in increments of 1 to 5 μl/min in the beginning, and then decrease the increments as levels approach euglycemia to ensure that 'overshooting' does not occur.

    An acceptable range of plasma glucose is within ±15% of 100 mg/dl (5.5 mmol/liter), i.e., from 85 to 115 mg/dl (4.68 to 6.33 mmol/liter). In general, the mice should reach stable euglycemia within 60 min. From this point on, the glucose infusion rate should not change much, and the animal is thus clamped.

14. Once euglycemia is reached, continue infusing glucose and insulin until $T = 180$ min, performing glucose measurements every 10 min instead of every 5 min.

15. At the end of the experiment, disconnect the mice and inject ~100 μl of saline into the catheters. Disconnect the glucose and insulin lines, purge them with water, and allow them to dry.

16. Place the mice in clean cages, provide food and water, and ensure that the mice recover from this stressful experiment.

    When the mice are finally euthanized, canulae can be recovered and purged with 70% ethanol for reuse.

Analyze data

17. For qualitative purposes, draw a curve that shows the progression of the clamp, i.e., plasma glucose versus time.

    This is especially useful for visualizing the stabilization at euglycemia during the last 60 min of the clamp.

18. Calculate GIR (mg glucose/kg/min) for each time point where glucose was sampled using the following equation:

$$\mathrm{GIR} = \frac{\mathrm{Glc}}{m} \times \mathrm{gir}$$

    where Glc is glucose concentration (i.e., 20 mg/100 μl or 20%), m is animal weight (kg), and gir is the glucose infusion rate (μl/min).

    If the gir was 5 μl/min and the mouse was 20 g (0.02 kg), then GIR = (20 mg/100 μl) ÷ (0.02 kg) × (5 μl/min) = 50 mg/kg/min.

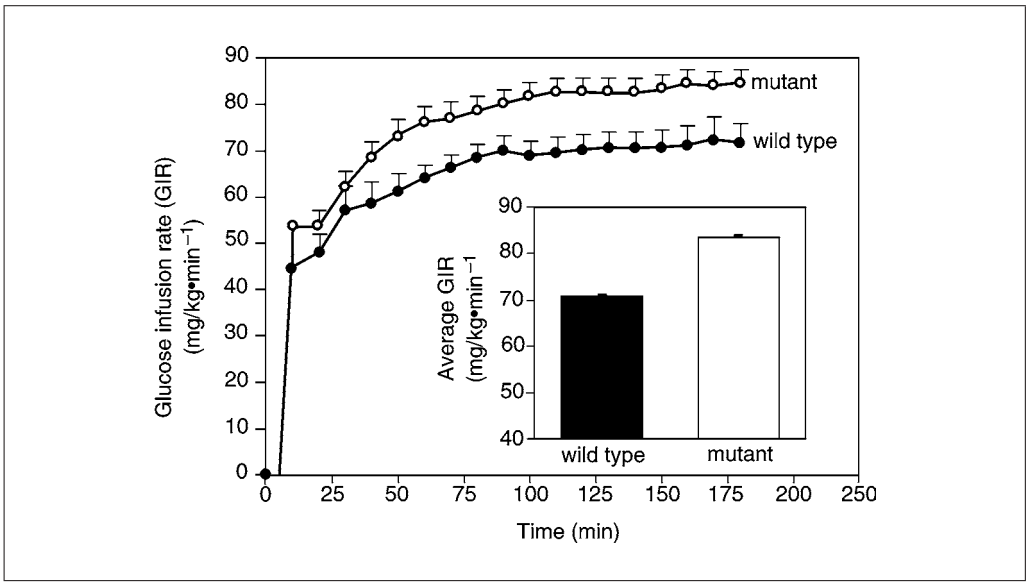

Figure 29B.3.4 Hyperinsulinemic-euglycemic clamp in wild-type and mutant mice. The evolution of glucose infusion rates (GIR), and the average thereof, show that the mutant mice are more insulin sensitive, being able to clear the infused glucose into peripheral tissues more rapidly.

19. Plot GIR versus time (see Fig. 29B.3.4).

The final result represents the GIR averaged over the last 60 min of the clamp, i.e., from 120 to 180 min, and is often depicted as a bar graph (inset in Fig. 29B.3.4).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Glucose solution, 20% (w/v)

Dissolve 20 g D-glucose in 100 ml distilled water or saline. Filter sterilize using a 0.45-μm filter. Store up to 1 week at 4°C or 6 months at −20°C.

Insulin solution for euglycemic clamp

Diluent stock solution: Dissolve 50 mg fatty acid–free bovine serum albumin (0.5% BSA final) in 9.9 ml sterile water, and add 100 μl of 1 M HCl (10 mM HCl final). Filter sterilize using a 0.45-μm filter. Prepare fresh, or store in 1.5-ml aliquots up to 6 months at −20°C.

Working solution: Add 1.15 ml stock solution into 6.048 ml saline. Add 20.1 μl of 100 IU/ml Humulin insulin (Eli Lilly; 0.278 IU/ml final). Prepare immediately before use.

Insulin solution for IPIST

Diluent stock solution: Dissolve 50 mg fatty acid–free bovine serum albumin (0.5% BSA final) in 10 ml sterile water, and add 10 μl of 1 M HCl (1 mM HCl final). Filter sterilize using a 0.45-μm filter. Prepare fresh, or store in 1.5-ml aliquots up to 6 months at −20°C.

Working solution: Add 1.15 ml stock solution into 6.049 ml saline. Add 10 μl of 100 IU/ml Humulin insulin (Eli Lilly; 0.139 IU/ml final). Prepare immediately before use.

COMMENTARY

Background Information

A principle hormone in metabolic regulation is insulin, which acts to lower blood glucose by stimulating glucose uptake by skeletal muscle and fat and other tissues, and by inhibiting glucose production by the liver. Post-prandial surges and inter-prandial declines of insulin levels tightly control glucose levels such that both hypoglycemia and hyperglycemia, which are intolerable for most tissues, are prevented. One key to glucose control is the ability of pancreatic β-cells to react quickly to changes in blood glucose by altering insulin secretion.

The motivation for investigating insulin and glucose signaling in the mouse is related to the epidemic status of T2DM. The pathophysiological events leading to T2DM in the context of the metabolic syndrome in humans include β-cell failure and resistance of target tissues to insulin. When tissues become insulin resistant, organs cannot respond properly and hyperglycemia prevails. One molecular mechanism thought to contribute to insulin resistance in obese individuals is the increased availability of free fatty acids (FFA) due both to reduced capacity to store the FFA in adipose tissue and to increased lipolysis. In the muscle, increased FFA oxidation leads to reduced glucose oxidative utilization in part through the inhibition of enzymes like pyruvate dehydrogenase, required for the oxidation of glucose, and phosphofructokinase (PFK), a key glycolytic enzyme. Glycogen synthesis is also inhibited by elevated FFA levels, thereby reducing non-oxidative glucose utilization. In the liver, FFA affects the balance between glycolysis and gluconeogenesis. This is attributed to the tendency of FFA to increase glucose production by enhancing the expression of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase. The pancreatic β-cells initially try to compensate for the reduced insulin sensitivity by inducing insulin secretion further. Although the resulting hyperinsulinemia is a short-term solution, in the long run, the vicious cycle leads to β-cell dysfunction and severe peripheral insulin resistance (Fig. 29B.3.5).

Thus, there are several indicators of insulin resistance, including elevated fasting glucose and insulin levels and impaired glucose tolerance. These pathophysiological phenomena can be detected in the mouse using similar methods used in humans, including measurements of fasting glucose and insulin levels, glucose/insulin sensitivity tests, and more invasive clamps. After measuring circulating insulin and glucose levels at steady state, the most common test is to administer a glucose load and follow the homeostatic response, which is the culmination of an increase in insulin followed by insulin-dependent processes that lower glucose. Glucose tolerance then becomes an expression of the efficiency with which homeostatic mechanisms restore glycemia to basal levels after perturbation. Finally, the most technically challenging test is the glucose clamp, which is considered the gold standard because its interpretation is based on the least number of assumptions since glycemia is fixed under hyperinsulinemic conditions. The literature shows that correlations between these diverse measures of insulin sensitivity and glucose tolerance are often quite good.

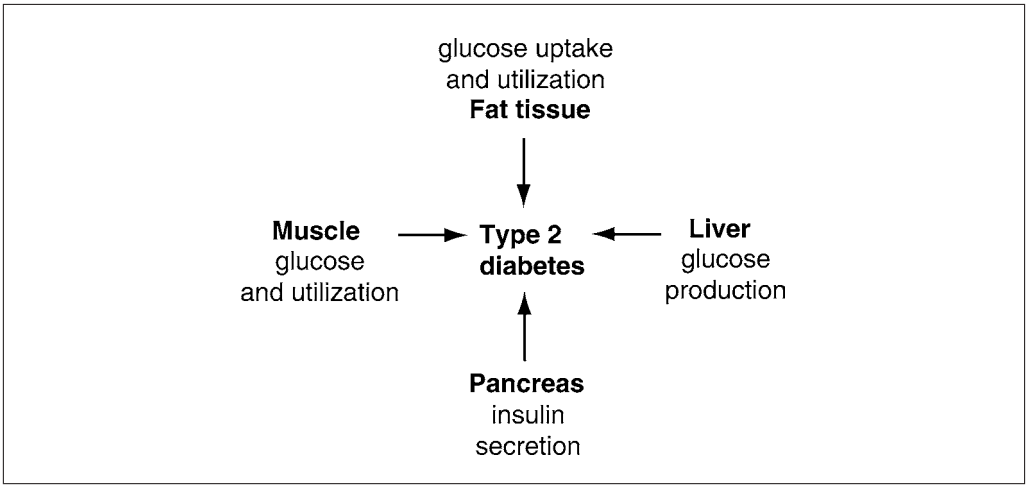

Figure 29B.3.5 Major effects of insulin resistance in different tissues, contributing to the development of type 2 diabetes mellitus (T2DM).

Critical Parameters

The validity of the results obtained from metabolic exploration phenotyping is largely dependent on methods of animal husbandry. To reduce the anxiety levels of the mice prior to and during testing, it is of vital importance that individuals performing these procedures are experienced, aware of the welfare of the mice, and familiar with the mice being tested. General laboratory procedures, and those specifically applicable to animal experimentation, should be followed throughout the protocols.

The majority of mouse metabolic studies are age, sex, and/or strain dependent (Champy et al., 2004). Therefore, it is important to keep these parameters consistent throughout a single experiment. It is also very important that all phenotyping experimentation be conducted at approximately the same time of day because physiological and biochemical parameters change throughout the day. Fasting should be done under conditions where the mice truly cannot eat, e.g., there are no fallen pieces of food pellets within the bedding material.

In tests where glucose is first administered and then measured in the blood, care should be taken not to contaminate any surfaces or materials with the glucose solution itself, as even a minute carryover into the blood samples will result in falsely high glucose measurements. Glucose syringes should be prepared cleanly and in advance, and any spillover or leakage from the injection site during the test should be carefully removed. Changing gloves after any suspected glucose contamination is recommended.

Since the changes in blood glucose (and insulin) concentrations during the tolerance tests are dynamic in nature, the number of mice that will be analyzed in any one experiment must be small enough that the sampling timetable can be precisely kept, especially during the critical initial phases where the most rapid changes are usually observed (typically T_0 to T_{30}). Although increasing the number of experimenters would facilitate the use of more mice in one experiment, for consistent results it would be best if one person obtains all blood samples. Using individual cages will aid in keeping the timepoints accurate by facilitating ease of mouse identification. Another practical method is to identify group-housed mice by marking their tails at the beginning of the test with a felt-tip pen.

Although using the composite measure AUC (or AUC_i) circumvents some of the statistical problems inherent with non-independent repeated measurements, it is still an approximation based on a fairly limited sampling of the dynamic state. Additional timepoints at the initial phase of the tests would make AUC more accurate, but this may lead to overbleeding the mice, or compromise timely sampling. Alternate methods for calculating the AUC are available, potentially giving more precise approximation from the same number of data points, but these require sophisticated mathematical methods and specialized software beyond the scope of this unit.

Glucose measurement by blood glucose monitor. Although blood glucose monitors provide accurate readings in most situations, it is advisable to use alternate methods to confirm very low readings (<36 mg/dl or 2 mM), which are possible during IPIST or initial phases of the euglycemic clamp, and especially very high values (reported upper limit for Accu-Check glucose monitor is 600 mg/dl or 33.3 mM), which are most likely to occur during peak phase of IPGTT. In addition, some mouse models for IR or T2DM such as KK-Ay mice have high enough glucose levels to very likely require alternate methods for blood glucose analysis.

Insulin measurement by ELISA. Since the overnight fasting insulin levels of the average mouse (see Anticipated Results) are relatively close to the sensitivity limit of even the ultrasensitive insulin ELISA kits (~0.2 μg/liter for the Mercodia kit), it is important to pay attention to proper sample storage conditions, minimization of freeze-thaw cycles of the samples, and good washing after the antibody incubation to obtain as many of the samples above the detection limit as possible. On the other hand, especially in some high-fat-fed diabetic mouse models, glucose-stimulated insulin levels may exceed detection limits (~7.0 μg/liter for the Mercodia kit), requiring dilution and re-analysis of such samples.

Oral glucose tolerance test. Successful oral gavaging is of vital importance to this test, and should be performed by experienced personnel to minimize animal loss due to gavaging the lungs rather than the esophagus.

Meal tolerance test. The mice should not be stressed before the feeding when free pellet feeding is used to avoid modifications in feeding behavior. For example, removing the bedding material or replacing it with paper towels is not advisable, even though accurate measurement of the food remaining at the end of the feeding period may be more difficult, since small pieces of food pellets may be missed within the bedding material.

Intraperitoneal insulin sensitivity and glucose tolerance tests. It is important to perform intraperitoneal injections in a manner that avoids leakage from the injection site. Another concern, especially with obese mice, is that the injected solution may end up as an inclusion within the large amount of adipose tissue, seriously slowing down the absorption of the substance and the metabolic effects thereof. Since ways to verify this are limited, especially if further experimentation on the same mice is desired, care should be taken at the data analysis phase to exclude unreactive mice. This will be particularly difficult if the groups of mice being compared have a significantly different amount of adipose tissue in the abdominal area, or if there are experimental groups that seem to be less reactive due to physiological rather than technical reasons. Thus, solid mouse handling and injection techniques are essential for success in these tests.

For some mouse models with increased insulin sensitivity, the insulin dose suggested in the protocol may be too high, resulting in severe hypoglycemia. Such mice must either be treated with an immediate injection of ~0.5 mg/g body weight glucose or be euthanized. A preliminary test with a small number of mice should be performed to verify the dose-responsiveness of the mice under study. On the other hand, in some experimental setups like after high-fat feeding, increasing the insulin dose may become a necessity. From a technical standpoint, it is also important that only freshly made insulin working solutions be used. This will prevent the insulin in the dilute solution from degrading or binding excessively to the walls of the tubes and syringes. Prior to each IPIST, it is recommended that the insulin first be tested on one additional mouse to verify that a fall in glucose is achieved at the 15-min timepoint.

Euglycemic clamp. Successful catheter placement is vital in this test, and the initial bolus injection of insulin serves to test this. Decreased glucose levels within 15 to 20 min indicate successful catheterization. In addition, care should be taken throughout the entire procedure to ensure that the external catheters are not blocked by tiny air bubbles or improper connections. If bubbles do arise, eliminating them by reconnections may be attempted, but experimentation on the affected mice should be stopped if the blockage is not cleared quickly.

The timing for blood sampling (one every 5 min) is to be used only as a guide. For example, when blood glucose concentration initially starts to fall, more frequent measurements can be taken to ensure that the animal does not become too hypoglycemic before glucose infusion can rescue it; this may lead to the loss of the animal. Similarly, once the animal is clamped and the plasma glucose is obviously stable, the measurements can become less frequent, e.g., one every 10 min. Care should be taken to minimize sample blood loss from the tail bleed to prevent an excessive fall in hematocrit.

It is also important to achieve and maintain euglycemia during the last hour of the test. In non-euglycemic conditions, changes in hepatic glucose output may interfere with the interpretation of the clamp data. In some cases, insulin infusion can be administered per unit of body surface area to avoid overinsulinization of obese mice.

Because of the stressful nature of the clamp experiment, further experimentation in the animals is not recommended, and the mice should preferably be euthanized. If other procedures such as tissue collection are vital, animals should be allowed to recover for at least 1 week.

Anticipated Results

Glucose and insulin measurements. Reference values for fasting plasma glucose and insulin values for a large cohort of C57B6/J mice are presented in Table 29B.3.2. However, depending on prior treatment (e.g., fasting, diet challenges, exercise), in combination with possible genetic modifications, blood glucose and especially insulin values can vary dramatically, particularly upwards. For example, mice lacking insulin receptor specifically in the liver have over 170 times more circulating insulin in the fed state than wild-type mice in the fasting state (Michael et al., 2000). Stimulation during glucose or insulin tolerance tests leads to an even wider range of variation. In models where IR is being induced, increased insulin is usually the first observed effect, and hyperglycemia may or may not follow. As such, elevated fasting glucose level indicates basal IR, which is most often attributed to impaired suppression of hepatic glucose output (gluconeogenesis and glycogen breakdown) by insulin, especially in cases where an increase in glucose is accompanied by an increase in

Table 29B.3.2 Fasting Plasma Glucose and Insulin Values in a Large Cohort of Female and Male C57Bl/6J Mice

	Mean ± SEM	*N*
Glucose (mg/dl)		
Female	115.5 ± 2.7	116
Male	123.6 ± 2.5	114
Insulin (μg/liter)		
Female	0.798 ± 0.120	70
Male	0.358 ± 0.072	78

insulin. On the other hand, low or normal insulin and high glucose levels in a fasting state is often indicative of advanced IR, including inadequate insulin secretion or even β-cell failure. Low insulin with normal glucose often indicates enhanced insulin sensitivity. In addition to conclusions drawn from these separate observations, HOMA indices for IR and β-cell function are useful for initial estimates of the phenotype and the validity of performing further, more invasive tests.

Tolerance tests in general. In all tolerance tests, excluding the IPIST, a considerable increase in glucose (and insulin, if measured) is observed in normal mice, followed by a decrease back toward basal level. Upon insulin challenge (IPIST), the reverse occurs for glucose. The amount and rate of increase and decrease in glucose (and insulin) vary widely depending on the mice and possible dietary or other challenges used. Some indication to possible ranges of outcomes is given below for each test, with a brief description of what the outcomes may indicate. In all tests, baseline (i.e., fasting) values for glucose (and insulin) should correspond to those obtained from prior blood samples from the mice in question.

Meal tolerance test. Changes in plasma glucose and insulin values upon consumption of a standardized meal not only gives information about the glucose homeostatic interplay of muscle, adipose tissue, liver, β-cells, and the brain, but also takes into account the signals that originate from the intestine upon nutrient absorption. Thus, the results of this test are best utilized when correlated with the results of other tests (e.g., IPIST and IPGTT) where intestinal absorption does not play a role. Typically, after a meal in normal mice, glucose and insulin values are expected to increase approximately two- and three-fold, respectively, compared to the fasting values. Food intake is normally in the range of 0.1 to 0.4 g of pellet chow.

Oral glucose tolerance test. Like the meal tolerance test, an oral dose of glucose will induce some intestinal effects; however, since only glucose is given rather than a full meal containing proteins, fats, and complex carbohydrates, this test scores only for intestinal glucose absorption. Typically, blood glucose is expected to increase 1.5 to 2-fold (T_{15}), followed by a slow decline towards baseline values. The evolution of blood glucose during the test often resembles that seen in the IPGTT (Fig. 29B.3.1A), although the peak is usually somewhat delayed and blunted and the decline back to baseline slower. In insulin resistant states, peak glucose is usually higher and, at a minimum, the following decline is delayed. An increase in the composite AUC measurement is typically a strong indication of stimulated IR, especially when calculated above baseline, as this eliminates the effect of any basal IR.

Intraperitoneal insulin sensitivity test. Excluding the euglycemic clamp, the intraperitoneal insulin sensitivity test is the most direct test of basal insulin sensitivity. Upon insulin injection, glucose typically falls from the fasting level until around T_{30}, after which it stabilizes and starts to climb back towards baseline upon degradation of the injected insulin (Fig. 29B.3.1B). In cases where FPG is greatly different between groups, plotting the glucose curve as percent of baseline values may be advantageous for visual representation, although this will be inherently accounted for in the calculations for the rate of initial decline and AUC_i using absolute values. The slower rate of decline in the initial phase of the test typically indicates impaired insulin sensitivity, mostly in skeletal muscle. During the later stages

of the test, especially at the final timepoints, slower recovery from the hypoglycemic state may indicate delayed activation of counter-regulatory signals or impaired hepatic glucose output. Optimally, all mice should react to insulin by lowered blood glucose levels that stay below baseline for the duration of the test. If this is not the case, calculating AUC_i becomes complicated due to difficulties in accomodating for those values that are higher than baseline. Care should be taken when analyzing the raw data to note such 'aberrant' values and possibly remove those mice from the analysis.

Intraperitoneal glucose tolerance test. Initial peak glucose values should be at least two-fold higher than the baseline value, but can be ten-fold higher or more, greatly depending on the particulars of the experimental set-up, including the genetic background of the mice as seen in Figure 29B.3.1A. When insulin is measured in conjunction with glucose, the evolution of that curve should resemble glucose, albeit with greater potential for variation. Increased AUC in an IPGTT indicates whole-body IR, in which skeletal muscle plays a major role. A significant increase in peak glucose (T_{15}-T_{30}) may be indicative of β-cell dysfunction; simultaneous insulin measurement is useful to support this.

Hyperinsulinemic-euglycemic clamp. During the clamp, insulin is administered as a constant infusion and therefore the inherent variations in endogenous insulin secretion are overwhelmed. Simultaneously, the plasma glucose concentration is clamped at the normal fasting level by means of an exogenous infusion of glucose. Thus, in vivo insulin action is measured under comparable conditions of stimulus and substrate. When the steady state is achieved, the exogenous glucose infusion rate equals the amount of glucose disposed of by all tissues, thus providing a quantification of overall insulin sensitivity. An increase in the glucose infusion rate implies increased insulin sensitivity, since the animal cleared more glucose for the same insulin concentration. For an example, see Figure 29B.3.4.

Time Considerations

General time considerations for metabolic phenotyping of mice have been described in *UNIT 29B.1*. Thus, only those considerations concerning specific protocols in this unit are given here.

Monitoring mouse blood glucose levels using glucometers designed for diabetic persons is very rapid, giving results within seconds. This is a prerequisite for performing more elaborate tests. Performing an insulin ELISA takes the better part of 1 day, but due to the microtiterplate format, hands-on time per sample is fairly short. The tolerance tests, excluding possible prior manipulations such as feeding the mice with altered diets, have a fixed time table. The only variability comes from the number of mice in each experiment. Excluding the overnight fasting, most of the tolerance tests take only a few hours to perform. The clamp is the most time-consuming procedure due to the requirement of catheter surgery and the small number of mice that can be experimented on at one time. Each blood sampling during the tests should not take >1 min. Analysis of the data from tolerance tests and the clamp is straightforward and fast due to the fairly small number of data points and the lack of complicated calculations. However, interpretation of the data may be a lengthy process.

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Histopathology in Mouse Metabolic Investigations

After establishing a metabolic phenotype in a mouse line through the use of in vivo phenotyping tests, the subsequent challenge is to refine this phenotype at the tissue, cell, and molecular (RNA and protein) levels. This is most easily achieved after sacrificing the mice. This unit focuses on histological analyses, but it should be noted that tissue sample size is often sufficient to allow for histopathological as well as molecular (RNA and protein) analyses.

This unit begins with a Strategic Planning section that addresses the overall strategic approach to histopathological evaluation, as well as specifics such as age and gender of the mice, cohort size, and controls. The four basic steps in histological analyses are fixation, embedding, sectioning, and staining. The protocols in this unit describe routine histological methods for tissue fixation (see Basic Protocols 1 through 7) and staining (see Basic Protocols 8 through 16). Paraffin embedding and sectioning are both described in *UNIT 14.1*. The staining methods start with a common and general histological method using hematoxylin and eosin (see Basic Protocol 8). This is followed by specialized histological stains used in the metabolic field, e.g., oil red O (see Basic Protocol 9) and osmium tetroxide (see Basic Protocol 10) for demonstration of intracellular lipids, detection of adipophilin (see Basic Protocol 11) for demonstration of intracellular lipid "ghosts," detection of cholesterol esters (see Basic Protocol 12), periodic acid Schiff (PAS) reaction (see Basic Protocol 13) for demonstration of polysaccharides, sirius red staining (see Basic Protocol 14) for demonstration of pathological collagen deposits, detection of succinate dehydrogenase in muscle tissue (see Basic Protocol 15) for staining of mitochondria, and en face staining of aortas with sudan IV (see Basic Protocol 16) for quantification of atherosclerotic plaques.

As histotechnology is time consuming and labor intensive, it is important to avoid unjustified experiments while achieving the highest degree of comprehensiveness. To this end, it is best to perform a first-line screen followed by organ/pathology-specific targeted assays. An in-depth evaluation of organs that have been fixed, processed, and embedded in a routine manner is used for first-line extended assays. Such assays involve ready-to-use tissue samples and are rapidly applied after completion of the systematic screen. In contrast, secondary assays correspond to in-depth evaluations of organs requiring special fixation, tissue processing, and/or embedding procedures, and thus the generation of a new cohort of mice.

A first-line histological screen is aimed at detecting the broadest array of tissue abnormalities in the mutant mouse line. Since each of the tissue defects represents a potentially invaluable clue towards understanding the in vivo functions of the gene under study, their inventory should be as exhaustive as possible. However, listing all the histological abnormalities in a given mouse is useless if they are not correlated with the genetic alteration. Thus, the overall goal of histopathology is to characterize the tissue lesions and ensure that they are caused by the genetic alteration. This latter goal can only be achieved if proper control mice are analyzed along with those bearing the genetic alteration.

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STRATEGIC PLANNING

Fixation methods

Once death has occurred, tissues undergo a process of self-digestion (autolysis) that is accelerated by heat and by post-mortem colonization of bacterial flora from the gut, respiratory system, and urinary tract (putrefaction). The first goal of fixation is to halt post-mortem tissue alterations, preserving cell structures in forms that resemble the living state. Fixation also hardens the tissue, which helps in subsequent handling. It is important to keep in mind that a given fixative stabilizes tissues in a reproducible manner by introducing a consistent tissue artifact, but that the particular changes in the appearance of cells and extracellular matrices are specific to each fixative. Unless otherwise specified, fixation methods are performed at room temperature. A summary of fixation methods can be found in Table 29B.4.1.

Staining methods

Hematoxylin and eosin (H&E) staining is the routine method performed in histology laboratories for paraffin-embedded tissue sections. Other stains are referred to as "special stains" because they are employed in specific situations according to diagnostic needs. Unless otherwise specified, staining methods are performed at room temperature. A summary of staining methods for metabolic analyses can be found in Table 29B.4.2.

Importance of performing systematic analyses

At least two scenarios can apply to mutant mouse lines entering a histological screen. The "black box" scenario corresponds to situations when the mutant mice have no overt clinical phenotype, when they die post-natally at variable ages for unknown reasons, and/or when the expression domain of the mutated gene is widespread and a large number of tissues are potentially targeted. In contrast, a mutant mouse line entering a systematic histological screen may display consistent clinical and/or histological defects

Table 29B.4.1 Summary of Fixation Methods for Histology

Fixation	Primary use	Advantages	Disadvantages
Formalin	Routine histology	Suitable for most stains; preserves some antigens	Unsuitable for testes, embryos, and fetuses
Bouin's fluid	Histology	Excellent morphological preservation of embryos, fetuses, and testes	Unsuitable for immunohistochemistry
Glutaraldehyde	Standard transmission electron microscopy	Preserves cellular ultrastructure	Unsuitable for paraffin sections and immunohistochemistry
Paraformaldehyde	Immunohistochemistry	Preserves antigens; permits good morphological preservation	Must be prepared fresh before each use
Perfusion	Immunohistochemistry, histoenzymology, and in situ hybridization	Most efficient delivery of fixative and quickest way to fix tissues and organs	Requires experienced manipulator and license for dissecting live animals
Freezing	Immunohistochemistry, histoenzymology, and in situ hybridization	Preserves antigens, fats, RNAs, and enzyme activity	Poor morphological preservation

Table 29B.4.2 Summary of Histological Stains for Metabolic Phenotyping

Stain	Primary use	Advantages	Disadvantages
Hematoxylin and eosin	Routine histological stain	Fast and reproducible	No specific staining of intra- or extracellular materials
Oil red O	Staining of neutral fats	Fast; enables semi-quantitative evaluation of neutral fats	Only applicable to sections of frozen tissues; does not distinguish triglycerides from cholesterol esters
Osmium tetroxide	Staining of unsaturated lipids	Sensitive; best method to detect small fat droplets	Highly toxic
Adipophilin immunostaining	Detection of protein coat of intracellular lipid droplets	Sensitive; applicable to routine paraffin-embedded histological sections	Time consuming and expensive
Acetic anhydride/ sulfuric acid stain	Detection of cholesterol esters	Distinguishes cholesterol esters from other intracellular inclusions	Reagents are toxic; only applicable to frozen tissue sections
Periodic acid Schiff	Detection of glycogen	Enables semi-quantitative evaluation of glycogen deposits	Pararosaniline (basic fuchsin) is carcinogenic
Sirius red	Detection of collagen fibers	Enables semi-quantitative evaluation of collagen deposits	Toxic and carcinogenic
Succinate dehydrogenase	Staining of mitochondria	Discriminates muscle fiber types on basis of their oxidative potential	Only applicable to sections of freshly frozen tissues
En face staining of aortas	Detection of atherosclerotic lesions	Enables quantitative measurement of atherosclerotic plaques	Requires an experienced manipulator

restricted to a single organ or to a small subset of organs expressing the gene under study. Not surprisingly, the affected organ(s) is usually in the field of expertise of the investigator who engineered the mutation. In a metabolic screen for instance, the tissues that are usually considered include the liver, brown adipose tissue, subcutaneous fat depots, paragenital fat pads, retroperitoneal fat pads, perirenal fat pads, pancreas, aorta, intestines, and muscles. However, looking only at selected organs can result in erroneous conclusions. Almost any mutant mouse is eligible for a systematic screen, even when it carries an organ-specific gene alteration generated through somatic mutagenesis. Altering the functions of a single organ may have broad and unexpected secondary consequences on the whole organism (Li et al., 2005).

Optimal age for analyzing a mutant mouse line

Mice are weaned and sexually mature at 3 and 6 weeks, respectively. Likewise, histogenesis is completed at 3 weeks in most tissues and at 6 weeks in reproductive organs. Although a 6-week-old mouse can be considered a young adult, necropsies and histological analyses are in general postponed until ~4 months, which is the time required for the completion of in vivo phenotyping tests. It should be stressed that a number of mutations affect the propensity to develop age-related diseases. Thus, although the cost of maintaining aging mice is prohibitive, it is imperative to investigate old (i.e., >12 months) and even senescent (i.e., > 24 months) subjects for late-onset diseases such as degenerative tissue changes and cancers (Harvey et al., 1993; Fan et al., 1998; Huang et al., 2002; Kim et al., 2002). Conversely, if the mutants die before 4 months of age, it will be necessary to determine the window of lethality before selecting an age group for morphological evaluation. It may be necessary to study the mutant line at several different ages to accurately follow the progression of the phenotypic changes and thus understand the functions of the mutated gene.

Gender

The question regarding the use of males or females in histological investigations is not trivial, as male physiology is not subject to the cyclical changes that, in females, can present another source of phenotypic variability. At first sight, one might imagine systematically analyzing males, and restricting the analysis of female organs to those exhibiting sexual dimorphism (reproductive organs, hypophysis, kidneys, and salivary glands). However, in mutant lines, gender-specific tissue alterations have been reported in organs that do not display sexually dimorphic histological features (Costet et al., 1998; Liu et al., 2000). Therefore, in a first-line histological screen, an equal number of males and females should be systematically analyzed.

Control mice and the importance of comparative histology

To interpret histological data, it is important to have a comprehensive knowledge of the phenotypic peculiarities and common lesions present in the inbred strains that are used to generate the experimental mouse line. The same holds true in respect to the environment of the experimental mouse line (pathogen status of the animal facility, chlorine or antibiotics in drinking water, and so on). This information must be taken into account to interpret possible discrepancies in the data stemming from different laboratories. Common pathologies of inbred strains are mentioned in Brayton et al. (2001) and Naf et al. (2002). It is mandatory, when designing a histological screen, to compare mutant mice to control mice that share the closest possible genetic background and are bred under identical conditions (for further discussion, see *UNIT 29A.2*).

Size of cohort and the first-line phenotyping strategy

The size of a mouse cohort should be sufficient to allow meaningful scientific interpretation of the data. Scientific statements are generally considered acceptable if they are characterized by an uncertainty $<5\%$ (significance, $p<0.05$). In this context, knowledge of the pathologies commonly encountered in the inbred strains composing the genetic background of the experimental mice is relevant in the consideration of group size. On a practical level, 24 mice per age group (six mutants of each gender and their controls) appears to be a reasonable number. However, as it takes ~4 hr of technician time to collect, process, and stain the tissues of a single mouse, economical considerations must be taken into account. Additionally, the benefit of a systematic histological analysis of ~40 different organs in 24 mice is uncertain for at least two reasons. First, it cannot identify morphological defects associated with subcellular components that are below the resolution level of light microscopes, or defects that are not detectable by routine hematoxylin and eosin staining. It is noteworthy that there are, in fact, relatively few features that can be unequivocally identified and quantified from hematoxylin/eosin-stained histological sections, i.e., cells/extracellular matrices, cavities/secretions, nuclei/cytoplasms, and cellular contours. Second, a systematic histological analysis will not ensure detection of all morphological defects that can be revealed by the routine stain, notably, defects that are heterogeneously distributed in an organ and/or occur at a low frequency in the mutant mouse line. These considerations highlight the need for a phenotyping strategy.

In the flow scheme shown in Figure 29B.4.1, each age group is subdivided into two subgroups on the day of sacrifice. Subgroup A consists of three mutants of each gender, one wild-type male, and one wild-type female. Mice belonging to this subgroup are subjected to a systematic necropsy (i.e., macroscopic analysis and weighing of selected organs) and to a systematic microscopic analysis of ~40 organs (see Table 29B.4.3). Following tissue dissection, all organs, with the exceptions of testis and long bones, are then fixed for a standard period of 24 hr in 10% (v/v) neutral buffered formalin prior

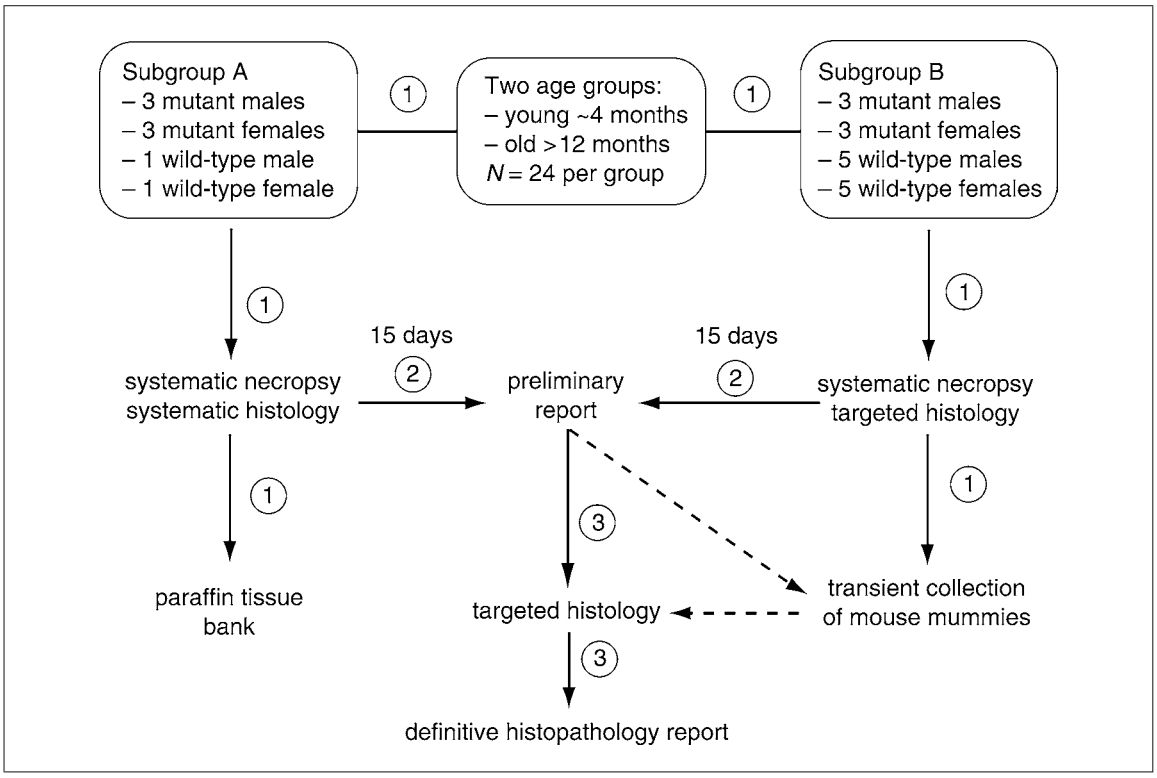

Figure 29B.4.1 Proposed flow scheme for processing tissues from a cohort of 24 mice. Step 1. Each age group is subdivided into two subgroups on the day of sacrifice. Mice from subgroup A are subjected to systematic macroscopic and microscopic analyses. What remains of the paraffin blocks after sectioning is archived in a paraffin tissue bank. Mice from subgroup B are subjected to systematic necropsy and to a targeted histological evaluation. At this point, the subgroup B organs destined for paraffin embedding are the ones carrying a macroscopically visible defect in at least one of the mutants. The remaining organs and the carcasses of the mice belonging to subgroup B are kept in buffered formalin until the completion of the histological screen (transient collection of mouse mummies). Step 2. A preliminary histopathological report will be available ~2 weeks after sacrifice. Step 3. Depending on the histological findings, a second round of targeted histological analysis of subgroup B mice may be undertaken with the objective of increasing the statistical significance of the preliminary observations. Organs of subgroup B analyzed by histology at this stage are the ones in which histological defects have been detected in the subgroup A mutants. To this purpose, these organs are collected from the bank of mouse mummies, processed, embedded, and then stained with hematoxylin and eosin.

to paraffin embedding. The testes are fixed in Bouin's fluid, which not only gives a more life-like preservation of the testicular parenchyma than buffered formalin, but also allows one to perform a high-contrast PAS stain, which is required to determine stages of the seminiferous epithelium cycle. Long bones (totally free from attached muscles) are fixed in formalin for 48 hr to prevent subsequent decalcification. For each organ, one slide is prepared carrying three consecutive 5-μm-thick histological sections, and is stained with hematoxylin and eosin. Therefore, only a small portion of the organ is evaluated systematically. What remains of the paraffin block is archived to generate a paraffin tissue bank that will be kept for years. These paraffin blocks can be used later for first-line extended assays, including serial sections through the organ, special histological stains, immunohistochemical stains, and cell proliferation and cell death assays. With respect to immunohistochemistry, it is expected that the duration of formalin fixation is short enough to preserve a large number of epitopes.

Subgroup B is comprised of the six remaining mutant mice and ten wild-type mice. Mice belonging to this subgroup are subjected to systematic macroscopic evaluation and a targeted histological evaluation. At this point, the organs that carry a macroscopically

Table 29B.4.3 Tissues Analyzed in a Systematic Necropsy Protocol

Functional system	Tissues
Cardiovascular system	Heart; aorta
Respiratory system	Trachea, lungs
Digestive organs	Liver, pancreas (exocrine), salivary glands
Digestive tract	Esophagus, stomach, duodenum, ileum, colon
Central nervous system	Brain
Sensory organs	Eye and adnexia, tongue
Endocrine system	Pituitary, thyroid, and adrenal glands, pancreas (endocrine)
Immune and haematopoietic system	Thymus, spleen, lymph nodes, bone marrow
Urinary system	Urinary bladder, kidney
Male reproductive system	Testis, epididymis, prostate, seminal vesicles, preputial glands
Female reproductive system	Ovaries, oviducts, uterus, vagina
Musculoskeletal system	Striated muscle, knee joint
Supporting and connective tissues	White and brown adipose tissue
Integumentary system and mammary gland	Skin and appendages, mammary gland

visible defect in at least one of the mutants in subgroup B are embedded in paraffin. For instance, if a liver nodule is observed in one mutant mouse, the livers of all twelve mutant mice and all twelve wild-type controls are immediately embedded in paraffin for subsequent histological analysis. The remaining, apparently intact, organs and carcasses of the mice belonging to subgroup B are kept in buffered formalin until the completion of the first-line histological screen. Following the ~2-week period required to complete the histological analysis of the subgroup A mice, a second round of targeted histological analysis of subgroup B organs is undertaken. In the second round, the organs of subgroup B that are analyzed by histology are the ones in which histological defects have been detected in the subgroup A mutants (Fig. 29B.4.1).

The first-line screen can lead to four possible conclusions. (1) Tissues from mutant mice, fixed in buffered formalin and stained with hematoxylin and eosin, appear normal. This result will end the analysis. (2) Mutant mice display morphological defects that are not correlated with the mutation, as they occur at the same penetrance and with the same expressivity as in control mice. These defects should be considered spontaneous "background effects" unrelated to the mutation. This will also end the analysis. (3) Mutant mice display morphological defects that are also present among control mice, albeit at a lower frequency. In this case, the first-line screen must be repeated on a larger sample to obtain statistically significant data. (4) Mutant mice display morphological defects that are correlated with the mutation. In this case, the organ defects should be analyzed further using first-line extended and secondary histological assays.

First-line extended assays

Organs entering these assays display known abnormalities that have, in principle, been related to the mutation. The objectives now are to refine the penetrance and expressivity (i.e., the variations in severity) of each defect in the mouse line, to refine the accuracy of

the diagnosis, and to gain insights into the molecular defects associated with the abnormal morphology. First-line extended assays are performed on tissues already collected and preserved during the first-line screening, and can involve any of the following. (1) Serial histological sections, collected consecutively or at regular intervals through the paraffin block. The goal is to increase the chances of lesion detection through analysis of organ volumes. (2) Histomorphometry aimed, for example, at determining the relative amount of Langerhans cells in the pancreas. These analyses can often be performed with the help of user-friendly, free software (e.g., ImageJ at *http://rsb.info.nih.gov/ij/download.html*). (3) Cell proliferation and cell death assays. (4) Immunohistochemical assays (e.g., immunodetection of lipid droplet "ghosts" with anti-adipophilin antibodies; Heid et al., 1998; Buechler et al., 2001; Mascrez et al., 2004). (5) Tissue and pathology-specific stains. In the metabolic field, these secondary assays include notably the PAS reaction (McManus and Mowry, 1960; Gabe, 1968) and sirius red staining for collagen fiber deposits (Junqueira et al., 1979).

The limits of the methods that can be applied in these assays are defined by the state of routinely processed organs. For instance, organs in paraffin are unsuitable for detecting certain cellular constituents, such as neutral lipids, that are extracted by the alcohol series and paraffin solvents. These organs are also unsuitable for electron microscopic studies, which require specific fixation and embedding procedures. Additionally, immunohistochemical analyses on these organs is not always successful, since antigens are often lost during formalin fixation, dehydration, and/or heating of tissues (Werner et al., 2000).

Secondary assays

Similar to the first-line extended assays, secondary assays are targeted to specific organs or organ systems. However, they require the use of special protocols for tissue collection (e.g., perfusion fixation) or special histological stains that cannot be adapted to formalin-fixed and paraffin-embedded tissues, and therefore require the generation of a new cohort of mutant and control mice. Secondary assays may include electron microscopic analyses as well as histochemical, immunohistochemical, and in situ hybrydization assays. In the metabolic field, notable secondary assays include oil red O stain (Lillie, 1965), detection of cholesterol esters (McManus and Mowry, 1960; Mascrez et al., 2004), detection of succinic dehydrogenase in muscle tissue (Carson, 1997), en face staining of aortas with sudan IV (Ganter and Jollès, 1970; Barnes and Weinberg, 1998), and electron microscopic analysis of mitochondria and other organelles (Maunsbach and Afzelius, 1999).

CAUTION: Many of the chemicals used in these protocols are hazardous in one or more ways (e.g., toxic, noxious, explosive, corrosive), and should be used only by personnel trained in appropriate protective measures. Refer to Material Safety Data Sheets for those chemicals for which specific information is not given within this unit.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

FIXATION WITH FORMALIN

BASIC PROTOCOL 1

Formalin (i.e., formaldehyde) stabilizes cell structures by introducing covalent cross-links between proteins. Formalin renders small proteins insoluble by linking them to structural proteins. It has no direct effects on lipids, carbohydrates, and RNAs, although it can preserve some of these components by trapping them in the cross-linked protein network.

CAUTION: Formalin is a carcinogen and an eye, skin, and respiratory irritant. Avoid contact and inhalation. Work under a well-ventilated chemical fume hood and wear disposable gloves. Collect used fixatives containing formalin in an appropriate waste container. For further information, consult a local center for occupational health and safety.

Materials

Mice (see Strategic Planning)
10% (v/v) neutral buffered formalin (equivalent to 4% [w/v] formaldehyde; e.g., Carlo Erba)

70% ethanol
Plastic histo-cassettes (e.g., Labonord)
Large (≥1 liter) containers with lids (e.g., Labonord)

1. Excise desired tissue and promptly place into a labeled plastic histo-cassette and close the lid. Do not overfill the cassette (the lid should close without resistance).
2. Immediately place the cassette in 10 to 20 volumes (relative to tissue volume) of 10% neutral buffered formalin in a large container and close the lid.

   Tissue should be placed in fixative preferably within 30 min to avoid autolytic alterations.
3. Fix for 20 to 24 hr at room temperature.
4. If tissues cannot be processed immediately, rinse 10 min in 70% ethanol and store in 70% ethanol at room temperature until use (up to 1 month).

   Do not reuse the buffered formalin.

   Formalin-fixed tissues can also be cryoprotected (see Basic Protocol 4, step 5b).

BASIC PROTOCOL 2

FIXATION WITH BOUIN'S SOLUTION

Bouin's solution is used routinely for mouse embryos, fetuses, placentas, as well as adult testes because it preserves nuclear details and chromosomes better than formalin and causes less tissue distortion (Fig. 29B.4.4). Bouin's solution is a mixture of formalin, picric acid, and acetic acid. Whereas formalin introduces covalent crosslinks between proteins, picric acid forms picrates with proteins, which are insoluble in alcohols. Compared to buffered formalin, Bouin's solution improves trichrome stains because of its mordanting effect on collagen fibers (Luna, 1992). However, it is unsuitable for immunohistochemistry, unless the antigen is very robust.

CAUTION: Dry picric acid is a shock-sensitive explosive capable of releasing energy on a level similar to dynamite. Dry picric acid should not be moved under any circumstances. Add water to the container of picric acid on a regular basis to prevent the product from drying out. For safety considerations for formalin, see Basic Protocol 1.

Materials

Mice (see Strategic Planning)
Bouin's fixative solution (e.g., Carlo Erba)
70% ethanol

Plastic histo-cassettes (e.g., Labonord)
Large containers with lids (e.g., Labonord)
Rocking platform

1. Excise desired tissue and promptly place into a labeled plastic histo-cassette and close the lid. Do not overfill the cassette (the lid should close without resistance).

2. Immediately place the cassettes in 10 to 20 volumes (relative to tissue volume) of Bouin's fixative solution in a large container and close the lid. Work as quickly as possible to avoid autolytic changes.
3. Fix for 24 to 48 hr at room temperature.
4. Wash in 70% ethanol for 1 hr at room temperature.
5. Wash again overnight under mild agitation on a rocking platform to remove as much picric acid as possible before embedding in paraffin.

   Proceed to embedding within 1 month.

FIXATION WITH BUFFERED GLUTARALDEHYDE

BASIC PROTOCOL 3

Glutaraldehyde is a dialdehyde and is therefore more efficient in cross-linking proteins and maintaining cellular ultrastructure than formaldehyde, which is a monoaldehyde. It is widely used for the production of thin sections (~50 to 70 nm) destined for standard electron microscopy, and of standard semi-thin sections (1 to 2 μm). Glutaraldehyde acts by cross-linking the polypeptide chains of proteins. It does not react with lipids. In contrast to formaldehyde, which penetrates tissues rather quickly due to its low molecular weight, glutaraldehyde penetrates the tissue rather slowly. Only small blocks of tissue (1 to 8 mm^3) are fixed well by immersion. It is therefore recommended to start fixing the tissue by perfusion with glutaraldehyde solution (Maunsbach and Afzelius, 1999). For a detailed perfusion method, see Basic Protocol 5. It must be stressed that tissues fixed with 2.5% glutaraldehyde are too hard to be sectioned properly in paraffin, and are also unsuitable for immunohistochemistry.

CAUTION: Glutaraldehyde is a carcinogen and an eye, skin, and respiratory irritant. For safe handling, see cautionary note for formalin in Basic Protocol 1.

Materials

Mice (see Strategic Planning)
2.5% (w/v) buffered glutaraldehyde in PBS (e.g., Sigma-Aldrich), freshly prepared
2- to 5-ml plastic tubes with lids

1. Perfuse the mouse with cold (4°C) 2.5% buffered glutaraldehyde (see Basic Protocol 5).

   Perfusion fixation is recommended, but tissues can be fixed without initial perfusion if this is not possible.
2. Remove the tissues of interest and trim them to ~2 × 2–mm cubes.
3. Transfer to ~10 vol of 2.5% buffered glutaraldehyde in 2- to 5-ml plastic tubes with lids. Leave for 1 hr at 4°C.
4. Inspect samples. If the fixative appears cloudy, replace it with fresh fixative.
5. Store fixed tissue in 2.5% buffered glutaraldehyde up to 12 months at 4°C.

FIXATION WITH BUFFERED PARAFORMALDEHYDE

BASIC PROTOCOL 4

Buffered paraformaldehyde is the most commonly used fixative for immunohistochemistry and in situ hybridization. It immobilizes protein antigens in tissues while retaining cell architecture and preserving epitope structure. Paraformaldehyde is a stable, solid form of formaldehyde composed of high-molecular-weight polymers. Upon heating, paraformaldehyde generates gaseous formaldehyde, which, when dissolved in water, reverts to the monomeric form. Paraformaldehyde solutions act by introducing covalent

cross-links between proteins. Compared to 10% neutral buffered formalin, freshly prepared 4% paraformaldehyde solutions do not contain contaminants, notably formic acid and methanol, which may destroy antigenicity (Polak and Van Noorden, 1997; Harlow and Lane, 1999; Allan, 2000). Tissues fixed in paraformaldehyde can be subsequently embedded either in paraffin or in tissue freezing medium.

CAUTION: Paraformaldehyde is a carcinogen and an eye, skin, and respiratory irritant. For safe handling, see cautionary note for formalin in Basic Protocol 1.

Materials

Mice (see Strategic Planning)
4% (w/v) buffered paraformaldehyde (PFA) fixative (see recipe)
1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)
70% ethanol
5%, 10%, and 20% (w/v) sucrose solutions in PBS
Razor blades

1. Remove the desired tissues, rinse in 1× PBS, and place into 10 to 20 vol of 4% buffered PFA fixative placed on ice. Work as quickly as possible to avoid autolytic changes. Leave tissues in fixative for 1 hr on ice.
2. Inspect samples. If the fixative is cloudy, replace it with fresh fixative.
3. Trim tissues into 3- to 5-mm-thick slices using a sharp razor blade to increase the surface area in contact with fixative. Place trimmed samples in 10 to 20 vol of fresh 4% PFA fixative.

   It is best to prepare these smaller slices after an initial fixation period to harden the tissues.

4. Fix for 6 hr (for soft tissue samples not thicker than 5 mm) to 12 hr, but never longer, at 4°C.

5a. *For embedding in paraffin:* Wash with several changes of 1× PBS for a total of 1 hr at 4°C, then store in 70% ethanol up to 1 month at 4°C until embedding.

5b. *For embedding in frozen specimen embedding medium:* Cryoprotect by using a sucrose gradient. Incubate tissues at 4°C as follows and proceed immediately to the freezing medium.

   30 min in 5% sucrose in PBS
   6 hr in 10% sucrose in PBS
   16 hr (overnight) in 20% sucrose in PBS.

   At each step, the specimen should fall to the bottom of the tube when properly infiltrated with the sucrose solution.

BASIC PROTOCOL 5

FIXATION BY PERFUSION

Fixation by perfusion provides the most efficient delivery of fixative and is thus the quickest way to fix tissues and organs, allowing optimal preservation of subcellular structures, antigens, and RNAs in situ. When samples are destined for immunohistochemistry at the light microscopic level or for in situ hybridization, the recommended perfused fixative is 4% buffered paraformaldehyde. For conventional electron microscopic observations, animals should be perfused with 2.5% buffered glutaraldehyde. Some investigators recommend a brief prewash of the vascular system to remove blood (to avoid intravascular clotting, which may hamper fixative perfusion), but this is unnecessary for most applications. Similarly, vasodilating substances may prevent vascular constriction, but are unnecessary in most cases.

This protocol is complementary to the one found in *UNIT 14.1*, where perfusion is performed on a euthanized mouse. In the authors' experience, performing the perfusion on a deeply anesthetized mouse, although somewhat more laborious, is more systemic since it takes advantage of the pumping heart to propel the fixative into circulation. It also avoids possible artifacts due to blood clots, which form rapidly in the capillaries of a dead animal.

CAUTION: Paraformaldehyde is a carcinogen and an eye, skin, and respiratory irritant. For safe handling, see cautionary note for formalin in Basic Protocol 1.

Materials

Mice (see Strategic Planning)
Anesthetic mixture (xylazine/ketamine; see recipe)
70% ethanol
Fixative: 4% (w/v) buffered paraformaldehyde (see recipe) or 2.5% (w/v) buffered glutaraldehyde in PBS (e.g., Sigma-Aldrich), freshly prepared
1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)

Cork board and pins
Dissection instruments (e.g., scissors, forceps)
Perfusion pump (e.g., Minipuls II, Gilson)
0.8-mm perfusion catheter (e.g., Tygon R3603 1/32 – 3/32)
0.5-mm perfusion needle (e.g., Terumo NN-2516R)
10- and 50-ml plastic tubes

Anesthetize mouse and expose heart

1. Anesthetize the mouse by i.p. injection of a lethal dose (0.3 ml) of anesthetic mixture. Wait until the mouse is under profound anesthesia (i.e., unresponsive to various stimuli, such as a foot pinch).
2. Lay the mouse on its back on a clean paper towel and pin all four extremities to a cork board. Wet the fur with 70% ethanol.
3. Open the abdomen with sharp scissors via a median incision from the pubic symphysis to the xyphoïd process of the sternum. Cut the skin and underlying muscles along the inferior border of the rib cage on both sides of the midline.
4. To access the thoracic cage, hold the inferior part of the sternum with forceps and cut the insertions of the diaphragm on the sternum and rib cage.
5. Fully expose the contents of the thoracic cage by cutting the ribs on both sides. Keep the sternum raised, and separate the insertions of its inner face with the parietal pleura and the pericardium. Take care not to damage any of the large thoracic vessels.
6. Recline the anterior wall of the thorax towards the head and pin it to the cork board (Fig. 29B.4.2A).

Perfuse mouse

7. Confirm the absence of air bubbles in the perfusion catheter and set the flow to deliver fixative drop by drop (not continuously) at ~3.5 ml/min. Insert the perfusion needle into the left ventricle (Fig. 29B.4.2B) and start the perfusion pump.
8. Quickly open the right auricle using sharp scissors to allow replacement of blood with fixative.

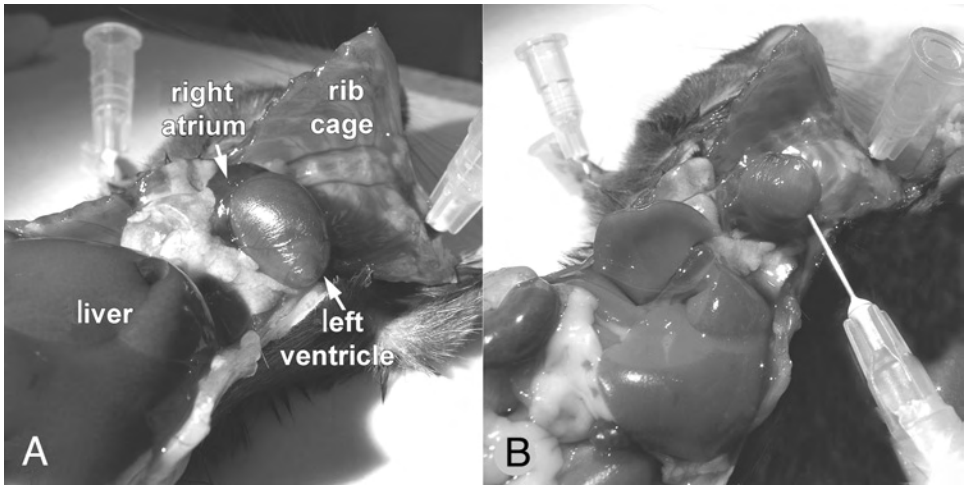

Figure 29B.4.2 Tissue fixation by perfusion. (**A**) The opened thoracic cage of a deeply anesthetized mouse is shown with the heart exposed. Note the reclined anterior wall of the thorax, pinned to the cork board on the right. (**B**) The correct insertion site of the perfusion needle into the left ventricle.

9. Evaluate perfusion efficacy according to the following criteria:

   a. The liver must turn from deep red to pale pink within 1 min. If the color of the liver does not change, reposition the needle in the left ventricle and restart the perfusion.

   b. The tail of the mouse must become stiff and the abdominal organs must harden.

10. Stop the perfusion after ~4 min (15 to 20 ml of fixative).

Dissect and fix tissues

11. Dissect the tissues of interest and place in 10- to 50-ml plastic tubes.

12. Fix the perfused tissues further by immersion in 10 to 20 vol of the same fixative solution for 6 to 16 hr at 4°C, depending on the size of the tissue sample.

13a. *For embedding in paraffin:* Wash paraformaldehyde-fixed tissues with several changes of 1× PBS for a total of 1 hr at 4°C, then store in 70% ethanol up to 1 month at 4°C until embedding.

Tissues destined for immunohistochemistry and in situ hybridization should not be stored in 4% paraformaldehyde.

13b. *For embedding in resin:* Store glutaraldehyde-fixed tissues in fixative for up to 12 months at 4°C prior to embedding.

14. Wash the catheter and perfusion needle with deionized water.

BASIC PROTOCOL 6

FREEZING TISSUES IN LIQUID NITROGEN VAPORS

Frozen sections are often used for histoenzymology, immunohistochemistry, in situ hybridization, and fat stains. Many antigens, enzymes, and RNAs do not survive routine paraffin embedding, and many lipids are extracted by paraffin solvents. An alternative to paraffin embedding is to freeze the tissue. Freezing hardens tissues so that the tissues can then be sectioned in a cryostat. Unfortunately, the drawback to frozen sections is poor morphology of the resulting tissue. The two protocols in this unit are applicable to unfixed tissues and tissues fixed in buffered paraformaldehyde (see Basic Protocol 4) or

periodate/lysine/2% paraformaldehyde and eventually cryoprotected by infiltration with 20% sucrose in PBS. Freezing in liquid nitrogen vapors is used when a large number of tissues must be collected over a short period of time and by a single person.

NOTE: Tissue freezing media are inert, water-miscible supports that only surround the tissues without penetrating them.

Materials

Liquid nitrogen
Tissue freezing medium (e.g., Shandon Cryomatrix from Thermo)
Mice (see Strategic Planning)
1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)
5%, 10%, and 20% sucrose solutions in PBS
Dry ice

Styrofoam box with lid
Aluminum foil
Plastic embedding molds (e.g., Leica)
Dissection instruments (e.g., scissors, forceps)
Petri dishes
Filter paper

Prepare materials

1. Fill a Styrofoam box about halfway with liquid nitrogen.
2. Using aluminum foil, build a rectangular "boat" with a flat bottom and ~2-cm high walls. Place the boat onto the liquid nitrogen (Fig. 29B.4.3A).
3. Close the lid of the Styrofoam box.
4. Label plastic embedding molds and fill them with tissue freezing medium, avoiding bubble formation. Remove any bubbles with a needle.

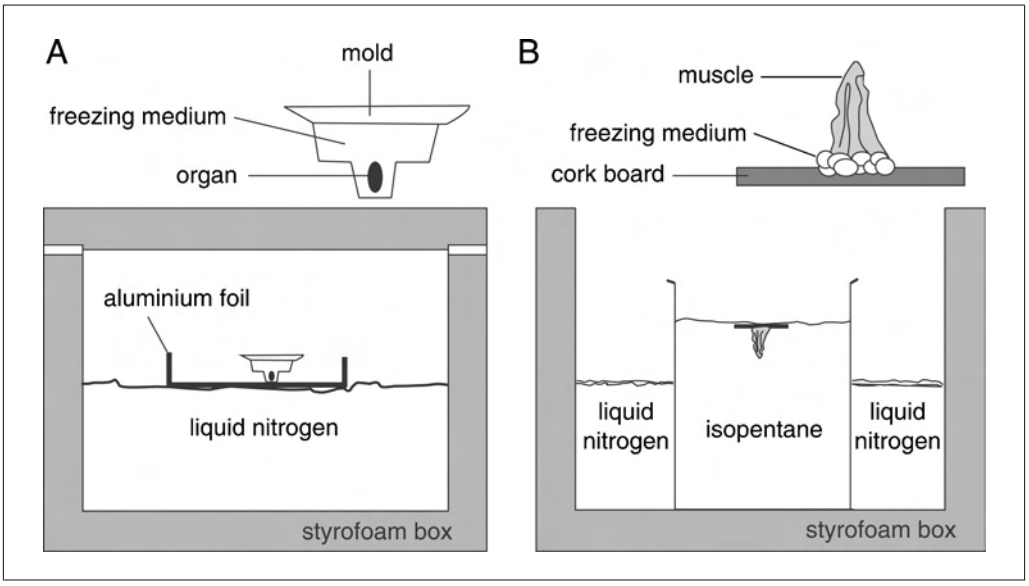

Figure 29B.4.3 Freezing tissue for histopathological purposes. (**A**) The correct positioning of an organ in freezing medium in the embedding mold for freezing in liquid nitrogen vapors. (**B**) The correct positioning of an organ in freezing medium on a piece of cork board for freezing in cold isopentane.

Dissect and pretreat tissues

5a. *For fresh tissue:* Dissect desired organs, using attached tissues such as ligaments and mesentery to handle the organs. Never squeeze the organ to be embedded. Remove as much as possible of the fat surrounding the organ, and rinse away any clotted blood in a Petri dish containing 1× PBS.

Fat watery tissues and tissues with heterogeneous textures will be difficult to section.

5b. *For prefixed tissue destined for immunohistochemistry:* Dissect organs, fix with 4% paraformaldehyde, and cryoprotect by using a sucrose gradient. Incubate tissues at 4°C as follows:

30 min in 5% sucrose in PBS
6 hr in 10% sucrose in PBS
16 hr (overnight) in 20% sucrose in PBS.

At each step, the specimen should fall to the bottom of the tube when properly infiltrated with the sucrose solution.

Embed and store samples

6. Blot the liquid surrounding the organ on filter paper.
7. Center the organ in the mold. Embed and orient the organ in the freezing medium, avoiding bubbles.

The organ should not touch any edge of the mold.

8. Transfer the mold onto the aluminum foil boat floating on the liquid nitrogen. Close the lid of the Styrofoam box.
9. Wait until the freezing medium turns completely white.
10. Collect frozen samples on dry ice, then transfer them to –80°C for long-term storage (up to 24 months).

BASIC PROTOCOL 7

FREEZING TISSUES IN ISOPENTANE

Freezing in isopentane represents the gold standard, but is less practical than freezing in liquid nitrogen (see Basic Protocol 6) for small organs or for procedures involving systematic collection and freezing of multiple organs. Skeletal muscle is an example of a tissue that needs to be frozen using cold isopentane to prevent the occurrence of ice crystal artifacts.

Materials

Mice (see Strategic Planning)
Tissue freezing medium (e.g., Shandon Cryomatrix, Thermo)
Liquid nitrogen
Isopentane (2-methylbutane; e.g., Sigma)
Dry ice

Dissection instruments (e.g., scissors, forceps)
1.5 × 1.5–cm pieces of cork board
Styrofoam box with lid
Glass beaker (cold-resistant)

1. Dissect unfixed muscle and place on a spot of tissue freezing medium on a 1.5 × 1.5–cm piece of cork board so that sections will be cut in a transverse direction (Fig. 29B.4.3B).

2. Place a small amount of liquid nitrogen (~5 cm deep) in a Styrofoam box.
3. Place a cold-resistant 1000-ml glass beaker containing 200 ml isopentane into the liquid nitrogen.
4. Wait until the isopentane is syrupy and almost solidified. If it does solidify, remove the beaker from the container and stir the isopentane with a spatula until it is mostly liquid again. When the isopentane has reached the appropriate temperature, place the glass beaker in dry ice to keep the isopentane temperature constant.
5. Plunge the cork square into the isopentane, tissue side up until the tissue freezing medium solidifies, then turn the cork board tissue side down.
6. Transfer to –80°C for storage (up to 24 months).
7. To section tissue, remove from freezer, allow to equilibrate in a cryostat chamber, and then attach the cork square to the cryostat stage using tissue freezing medium.

HEMATOXYLIN AND EOSIN STAINING

BASIC PROTOCOL 8

Hematoxylin is not a dye per se, but its oxidized product, hematein, can preferentially stain nuclei provided that it is combined with a mordant or link to the tissues. The mordant for hematein is a metal cation such as iron, aluminum, or tungsten. The combination of hematein and its metallic mordant becomes a dye that exhibits an affinity for chromatin. The mordant-dye combination is called a lake. Lakes formed by hematein are positively charged and thus exhibit a strong affinity for nucleic acids notably present in nuclei. Harris hematoxylin is a commercially ripened (oxidized) hematoxylin that, similar to other alum hematoxylins, uses aluminum as a mordant for hematein. Harris hematoxylin is usually used as a regressive stain: the sections are overstained, then decolorized in a dilute acid solution (the color shifts from blue/purple to pink/red). After decolorization, the sections are placed in a dilute alkaline "bluing" solution to reestablish the insoluble (blue-colored) dye, allowing differentiation of chromatin from cytoplasmic details. A thorough rinse is necessary after this step to avoid adversely influencing the cytoplasmic staining step.

Eosin reacts with proteins. Like most cytoplasmic stains, eosin is an anionic (negatively charged) dye with an affinity for positively charged proteins. Because of the terminal amino and carboxyl groups commonly found on proteins and some side chains of amino acids, proteins are amphoteric, i.e., they may be either positively or negatively charged. The charge is dependent on the pH. When the pH of the staining solution is below the isoelectric point of proteins (pH ~6.0), the proteins are positively charged. The maximum uptake of eosin by cytoplasmic proteins occurs when it is used at pH 5.0.

This procedure is recommended for histological sections from paraffin-embedded tissues cut at 5 to 7 μm, collected on gelatin-water, spread on glass slides, and dried in an oven to remove any trace of water. It can be performed either manually or by an automatic stainer.

CAUTION: Avoid contact with and inhalation of histological stains and paraffin solvents. Wear disposable gloves when handling histological stains and paraffin solvents. Wear safety glasses when manipulating concentrated acid and basic solutions. Manipulate paraffin solvents under a laboratory fume hood. Collect paraffin solvents in a specific waste container. For further information, consult the local center for occupational health and safety.

Materials

Histological sections on glass slides
Clearing agent (e.g., Histosol Plus; Shandon)
95% and 100% ethanol
Harris hematoxylin (e.g., Merck), freshly filtered (e.g., using Rapid flow filter paper, Macherey-Nagel)
Acid alcohol: 0.37% HCl in 70% ethanol
0.1% (w/v) eosin Y (see recipe)
Permanent mounting medium (e.g., Eukitt, Labonord)

Staining jars or automated slide stainer
Hot plate at 58°C
25 × 60–mm glass coverslips

Prepare solutions

1. Set up a series of staining jars (or an automated slide stainer) with the necessary solutions in a chemical fume hood.

   For example, deparaffinizing and rehydrating the slides will require seven jars: two with clearing agent, two with 100% ethanol, two with 95% ethanol, and one with water.

Deparaffinize and rehydrate sections

2. Dry the sections for ~5 min at 58°C on a hot plate until the paraffin is just melted.
3. Deparaffinize the sections by placing slides in two changes of clearing agent for 3 min each.
4. Rehydrate the sections as follows:

   two changes in 100% ethanol, 3 min each
   two changes in 95% ethanol, 3 min each
   one wash for 3 min in deionized water.

   Once every 200 slides, discard the first 95% and 100% ethanol baths, use the second baths as the "new first" baths, and replace the second baths with freshly made solutions.

Stain sections

5. Stain for 3 min in Harris hematoxylin.
6. Wash in running tap water for 3 min.
7. Decolorize in acid alcohol for 2 sec.
8. Wash in running tap water for 3 min to "blue" the hematoxylin.
9. Stain for 30 sec in 0.1% aqueous eosin Y.
10. Rinse in tap water for 30 sec.

Dehydrate, clear, and mount sections

11. Dehydrate sections in graded alcohols as follows:

    two changes in 95% ethanol, 2 min each
    two changes in 100% ethanol, 3 min each.

12. Clear sections in two changes of clearing agent, 2 min each.

    The slides can remain in clean clearing agent until coverslipped.

13. Clean the underside of the slide. Never allow the clearing agent on the sections to evaporate completely.
14. Mount the sections using 2 to 3 drops of permanent mounting medium and place a glass coverslip on the slide. Avoid bubble formation. If any bubbles do form, press the coverslip gently with a needle point to remove them.
15. Dry the slides overnight at room temperature on a flat surface.

OIL RED O STAINING FOR NEUTRAL FATS

BASIC PROTOCOL 9

Staining with oil-soluble dyes such as oil red O is based on the greater solubility of the dye in lipid substances than in the dye solvent (e.g., isopropanol). Oil red O staining is widely used to detect neutral fats in cells and tissues. However, it does not allow for the distinction between triglycerides and cholesterol esters, nor for the detection of phopholipid inclusions in cells. Cholesterol esters can be specifically detected using sulfuric acid/acidic anhydride staining (see Basic Protocol 12), and phospholipid inclusions by immunohistochemistry (Mark et al., 1992).

In neutral fat detection, oil red O staining is only applicable to sections of frozen tissues, either unfixed or, for best results, fixed in 4% paraformaldehyde or 10% formalin. However, it is also a well-established method for demonstrating lipofuscins (lipid-containing residues of lysosomal digestion), which, in contrast to neutral fats, survive treatment with solvents during paraffin processing.

Materials

Histological sections on glass slides
1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)
Oil red O staining solution (see recipe)
Vectashield mounting medium (Vector Laboratories) containing 10 μg/ml DAPI (e.g., Roche; store in dark at 4°C)
Harris hematoxylin (e.g., Merck), freshly filtered (e.g., using Rapid flow filter paper, Macherey-Nagel), optional
Glycerol/PBS aqueous mounting medium: 9:1 (v/v) 87% glycerol/PBS (store at 4°C), optional

25 × 60–mm glass coverslips
Staining jars (e.g., Labonord)

1. Remove histological sections on glass slides from –80°C freezer and allow them to dry for 10 min at room temperature.
2. Rehydrate the histological sections and remove the freezing tissue medium by placing the slides in 1× PBS for 2 min.
3. Blot all excess 1× PBS using tissue paper. Do not allow the sections to dry.
4. Stain in oil red O working solution for 3 min.
5. Rinse in deionized water for 30 sec.
6. Mount sections in Vectashield containing DAPI (recommended).

   Alternatively, stain the nuclei by dipping in Harris hematoxylin for 1 sec, then wash two times in tap water for 2 min, and mount the slides in 78% glycerol/PBS.

7. Store stained slides horizontally at 4°C, where they can be kept for up to 12 weeks.

BASIC PROTOCOL 10

OSMIUM TETROXIDE STAINING OF UNSATURATED LIPIDS

Osmium tetroxide (OsO_4) is used more as a fixative for electron microscopy than as a stain, but it is still one of the best methods for revealing small fat droplets. It binds at double bonds of unsaturated lipids, rendering them insoluble in alcohols and organic solvents, and imparting a dense brownish or black color. Owing to the health hazard of osmium tetroxide, this procedure is usually applied only to tissues destined for electron microscopy (and thus fixed in 2.5% buffered glutaraldehyde). However, the blackening of lipids caused by osmium tetroxide is readily visible under light microscopy in semi-thin sections from resin-embedded tissues.

CAUTION: Osmium tetroxide is highly toxic and a rapid oxidizer. Severe reactions may result through all routes of exposure: inhalation, ingestion, contact with the eyes and other mucous membranes, and contact with skin. Because of its volatility, the vapor hazard is usually emphasized. Exposure to the vapor can cause severe chemical burns to the eyes, skin, and respiratory tract. Among the most insidious effects of osmium tetroxide is its capacity to cause irreversible blindness. Always manipulate osmium tetroxide under a well-ventilated chemical fume hood and wear gloves and other appropriate protective gear.

Materials

2 × 2–mm glutaraldehyde-fixed tissue cubes (see Basic Protocol 3)
1× phosphate-buffered saline (PBS, e.g., Sigma-Aldrich)
4% aqueous OsO_4 (e.g., Fluka)

2-ml microcentrifuge tubes
Aluminum foil

1. Wash glutaraldehyde-fixed tissue cubes two times for 5 min each in 1× PBS in 2-ml microcentrifuge tubes.
2. Prepare a 1% OsO_4 solution by diluting 1 vol of 4% stock solution in 3 vol of 1× PBS.
3. Incubate the samples in 1% OsO_4 1 hr at 4°C in complete darkness (wrap the microcentrifuge tubes in aluminum foil).
4. Wash two times for 5 min each in 1× PBS.
5. Dehydrate and embed for electron microscopic analysis. See, e.g., Maunsbach and Afzelius (1999) for techniques associated with electron microscopy.

BASIC PROTOCOL 11

IMMUNODETECTION OF LIPID DROPLET "GHOSTS" WITH ANTI-ADIPOPHILIN ANTIBODIES

Adipophilin (Adfp; or adipose differentiation related protein, Adrp) is associated with the surface of lipid droplets in a wide range of cells including hepatocytes and macrophages. It is a specific and sensitive marker for lipid accumulation in diverse diseases (Heid et al., 1998; Londos et al., 1999; Buechler et al., 2001; Mascrez et al., 2004). Immunodetection of adipophilin is used to detect the protein coat of intracellular lipid droplets. As this detection is applicable to histological sections fixed in 10% buffered formalin and embedded in paraffin, it offers an interesting alternative to the use of a lipid-soluble dye or osmium tetroxide, which require specific fixation and/or embedding procedures. Note that only the contours of lipid inclusions will be revealed, as the lipids themselves are dissolved by paraffin solvents during tissue processing.

Materials

1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)
1× PBS with 0.05% Tween 20 (PBST)
Histological sections, preferably on Superfrost coated glass slides (e.g., Labonord)
0.01 M sodium citrate buffer
Guinea pig polyclonal anti-adipophilin antibody (Research Diagnostics)
Cy3-conjugated anti–guinea pig antibody (Chemicon International)
Vectashield mounting medium (Vector Laboratories) containing 10 μg/ml DAPI (e.g., Roche; store in dark at 4°C)

Staining jars (e.g., Labonord)
Diamond-tip pencil
250-ml plastic container with lid for slide holder
Plastic slide holder
Humid chamber (24 × 24 × 2–cm plastic box with opaque walls containing a water-soaked tissue)
25 × 60–mm glass coverslips
Fluorescence microscope equipped with DAPI and Cy3 filters
Additional reagents and equipment for deparaffinization and rehydration (see Basic Protocol 8)

1. Set up a series of staining jars for deparaffinization and rehydration in a chemical hood, and for PBST and PBS washes on the benchtop.
2. Deparaffinize and rehydrate histological sections (see Basic Protocol 8, steps 2 to 4).
3. Place the sections against a black background and mark a circle around each section with a diamond-tip pencil to facilitate visualization.
4. Immerse the slides completely in 0.01 M sodium citrate buffer and heat 10 min in a microwave oven at 900 W (the solution must boil). Make up the original level of buffer with warm water, and cool to room temperature for 20 min.

   Heating unmasks epitopes that have been hidden by fixation.

5. Rinse 3 min in 1× PBS at room temperature. Wipe the PBS away completely around each section, but do not allow the sections to dry.
6. Cover each section with ~20 μl of 1:100 guinea pig polyclonal anti-adipophilin antibody in 1× PBS and incubate in a humid chamber for 16 hr at 4°C.
7. Rinse three times, 10 min each, in PBST and then one time for 3 min in PBS.
8. Incubate with 1:400 Cy3 anti–guinea pig secondary antibody in 1× PBS for 1 hr at room temperature in a humid chamber in the dark.

   Avoid any unnecessary exposure of the fluorescently labeled tissues to light.

9. Rinse three times, 10 min each, in PBST and then one time for 3 min in PBS. Wipe the PBS away completely around each section, but do not allow the sections to dry.
10. Under dim light, cover each section with a drop of Vectashield containing DAPI and mount under a 25 × 60–mm glass coverslip.
11. Store sections horizontally in the dark at 4°C.

    Sections can be stored for at least 1 week (more if sealed, e.g., with nail polish or a plastic sealant, around the perimeter of the coverslip).

12. Examine sections under a fluorescence microscope equipped with DAPI and Cy3 filters.

BASIC PROTOCOL 12

STAINING FOR CHOLESTEROL ESTERS

This staining procedure is used in tissue sections to detect inclusions containing cholesterol esters, distinguishing them from inclusions containing other lipids. It is based on Romieu's adaptation to histochemical use of the Liebermann-Burchard reaction for cholesterol and its esters (Kenny, 1952; McManus and Mowry, 1960). This procedure is applicable only to frozen tissue sections obtained from unfixed tissues or from tissues fixed in 4% paraformaldehyde in PBS.

CAUTION: Acetic anhydride and sulfuric acid are poisonous. Wear safety glasses and work under a laboratory hood throughout this procedure.

Materials

Tissue samples, fixed (see Basic Protocol 4) or frozen (see Basic Protocol 6)
10% neutral buffered formalin (equivalent to 4% [w/v] formaldehyde; e.g., Carlo Erba)
Concentrated sulfuric acid
Acetic anhydride
Permanent mounting medium (e.g., Eukitt, Labonord)

Cryostat
Glass microscope slide
Glass coverslips

1a. *For fixed tissue:* Cut 10-μm frozen sections using a cryostat (see *UNIT 14.2*), collecting each section directly on a separate drop of deionized water on a glass slide.

1b. *For unfixed tissue*: Cut 10-μm frozen sections using a cryostat (see *UNIT 14.2*), collecting each section on a drop of 10% neutral buffered formalin on a glass slide. Fix for 10 min. Wash three times by replacing fixative with deionized water and allowing the section to sit for 3 min.

2. Blot away as much of the water as possible without allowing the section to dry and adhere to the glass slide.

3. Cover the section with a drop of concentrated sulfuric acid and leave for 15 sec.

4. Add 3 drops of acetic anhydride.

5. Mount with a drop of mounting medium under a glass coverslip and examine immediately.

BASIC PROTOCOL 13

PERIODIC ACID SCHIFF STAINING FOR GLYCOGEN

Periodic acid Schiff (PAS) staining is used to detect glycogen and other carbohydrates in cells and extracellular matrices on histological sections. Aqueous periodic acid oxidizes 1,2-glycol groups in tissue components that consist of or contain carbohydrates, producing aldehydes that can be detected by Schiff's reagent. Schiff's reagent combines chemically with aldehydes to form a red product. Schiff's reagent is made from pararosaniline by treating it with sulfurous acid (H_2SO_3). The protocol is performed on tissues fixed in 10% neutral buffered formalin and embedded in paraffin, and can be performed either manually or by an automatic stainer.

CAUTION: Pararosaniline (basic fuchsin) is a known carcinogen. Work under a chemical fume hood and wear appropriate protective gear.

Materials

Histological sections on glass slides
0.5% (w/v) aqueous periodic acid (H_5IO_6)
Schiff's reagent (e.g., Merck or see recipe)
Harris hematoxylin (e.g., Merck), freshly filtered (e.g., using Rapid flow filter paper, Macherey-Nagel)
Acid alcohol: 0.37% HCl in 70% ethanol

Ultraclean staining jars
Glass coverslips
Automatic slide stainer (optional)

Additional reagents and equipment for deparaffinization, rehydration, clearing, and mounting (see Basic Protocol 8)

1. Set up a series of staining jars with the necessary solutions in a chemical hood.
2. Deparaffinize and rehydrate sections (see Basic Protocol 8, steps 2 to 4).
3. Place the histological sections on glass slides in 0.5% aqueous periodic acid for 5 min.
4. Rinse in tap water and then deionized water for 5 min each.
5. Place in Schiff's reagent for 15 min.
6. Rinse two times in deionized water for 5 min each.
7. Counterstain by dipping in Harris hematoxylin for 2 to 3 sec.
8. Wash in running tap water for 3 min.
9. Decolorize by dipping in acid alcohol for 2 sec.
10. Wash in running tap water for 3 min to blue the hematoxylin.
11. Dehydrate, clear, and mount sections (see Basic Protocol 8, steps 11 to 15).

SIRIUS RED STAINING FOR COLLAGEN FIBERS

BASIC PROTOCOL 14

This protocol describes the detection of collagen fibers on histological sections using Sirius red stain, which is a strong anionic dye and stains collagen via a reaction between its sulfonic acid groups and the basic groups of collagen. The elongated dye molecules are attached to the collagen fiber so that their long axes are parallel, which results in enhanced birefringency (i.e., the property of biological crystals to transmit light unequally in two directions) of these fibers (Junqueira et al., 1979). Sirius red staining enables morphometric (quantitative) measurement of collagen (Malkusch et al., 1995). Prior to staining, tissues are fixed in 10% neutral buffered formalin or Bouin's solution and embedded in paraffin.

CAUTION: Sirius red is a disazo dye. Little is known about the hazards associated with it, but other disazo dyes are toxic and carcinogenic. Handle with care. See Basic Protocol 2 for safety considerations about picric acid.

Materials

Histological sections on glass slides
0.1% picro-Sirius red staining solution (see recipe)
Acidified water: 0.5% (v/v) glacial acetic acid

Additional reagents and equipment for deparaffinization, rehydration, clearing, and mounting sections (see Basic Protocol 8)

1. Set up a series of staining jars with the necessary solutions in a chemical hood.
2. Deparaffinize and rehydrate histological sections on glass slides (see Basic Protocol 8, steps 2 to 4).
3. Stain sections 1 hr in 0.1% picro-Sirius red staining solution.
4. Wash two times in acidified water for 30 sec each.
5. Dip in deionized water and remove excess water by vigorously shaking the slide.
6. Transfer immediately to 100% ethanol (three changes, 1 min each).
7. Clear and mount sections (see Basic Protocol 8, steps 12 to 15).

BASIC PROTOCOL 15

SUCCINATE DEHYDROGENASE STAINING OF MITOCHONDRIA

Succinate dehydrogenase (SDH) is located in the inner membrane of mitochondria and catalyzes the oxidation of succinic acid to fumaric acid in the citric acid cycle. The histochemical detection of SDH activity specifically highlights mitochondria and is commonly used to discriminate muscle fiber types on the basis of their oxidative potential. Fibers with a high oxidative capacity generate ATP via oxidative phosphorylation in the mitochondria. Muscle cells that are rich in mitochondria have a higher oxidative capacity. Therefore, detection of SDH activity distinguishes oxidative fibers with high mitochondrial content (i.e., slow-twitch/oxidative type I fibers and fast-twitch/oxidative type IIA fibers are intensely stained) from nonoxidative (anaerobic) fibers with low mitochondrial content (i.e., fast-twitch/glycolytic type IIB and IIX fibers are poorly stained or unstained).

SDH activity is demonstrated by incubation of fresh tissue sections with succinate substrate in the presence of a tetrazolium compound such as nitroblue tetrazolium (NBT). The hydrogen released from the succinate substrate is transferred to NBT, which is reduced to a purple-blue pigment indicating the sites of enzyme activity (Nachlas et al., 1957).

Materials

Frozen histological sections on glass slides, freshly prepared
Succinate dehydrogenase staining solution (see recipe)
1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)
10% (v/v) neutral buffered formalin (equivalent to 4% [w/v] formaldehyde; e.g., Carlo Erba)
15% ethanol
Aqueous mounting medium (e.g., 90% glycerol/PBS)
Staining jars

1. Prepare a series of staining jars with the appropriate solutions.
2. Incubate slides in freshly made succinate dehydrogenase staining solution for 1 hr at 37°C.
3. Wash three times, 5 min each, in 1× PBS.
4. Postfix in 10% neutral buffered formalin for 10 min.
5. Rinse two times, 5 min each, in 15% ethanol.
6. Mount with aqueous mounting medium.

EN FACE STAINING OF AORTAS WITH SUDAN IV

En face staining of aortas is used to detect atherosclerotic lesions. Staining with oil-soluble dyes such as Sudan IV is based on the greater solubility of the dye in lipid substances than in the dye solvent. This procedure is applicable to whole aortas fixed by perfusion with 4% paraformaldehyde.

NOTE: Regulations concerning personnel allowed to perform intracardiac perfusions vary between countries. Consult the local authorities for the proper procedure.

Materials

Mice (see Strategic Planning)
4% (w/v) buffered paraformaldehyde (see recipe)
1× phosphate-buffered saline (PBS; e.g., Sigma-Aldrich)
70% and 80% ethanol
En face staining solution containing Sudan IV (see recipe)

Dissection instruments (e.g., scissors, forceps)
Dissecting microscope
15-ml tube
Blackened cork board
Insect pins (Fine Science Tools) or similar
Spring scissors (Fine Science Tools) or similar
Silicone-coated Petri dishes (see recipe)
Platform rocker

Additional reagents and equipment for perfusion fixation (see Basic Protocol 5)

Remove aorta and prepare for staining

1. Perfuse the mouse with 4% buffered paraformaldehyde (see Basic Protocol 5).
2. Remove all abdominal organs except the kidneys.
3. Perform this and all subsequent steps under a dissecting microscope. Dissect out the aorta with the heart by starting at the bifurcation of the aorta and continuing upwards to the heart. Take care not to tear the aorta at the level of the renal arteries or the diaphragm. Place the dissected aorta, still attached to the heart, in a 15-ml tube containing 10 ml of 4% neutral buffered paraformaldehyde. Allow to fix for at least 24 hr at room temperature.
4. Wash the aorta with the heart attached in 10 ml of 1× PBS for 3 min.
5. Pin the apex of the heart and the distal part of the aorta using insect pins on a blackened cork board.
6. Using microdissection scissors and fine forceps, remove all adipose tissue surrounding the aorta. Be cautious at the departure of branches not to damage the aorta.
7. Detach the aorta from the heart at the point where it enters the left ventricle.
8. Using fine forceps and scissors, open the aorta along its entire length.
9. Place the opened aorta, with the internal surface up, on a silicone-coated Petri dish. Using insect pins, pin the edges of the aorta along the entire length.

Stain aorta

10. Wash the aorta with 70% ethanol for 10 sec with gentle agitation. Discard the ethanol.

11. Cover the aorta with en face staining solution containing Sudan IV and allow it to stain with gentle agitation for 15 min. Discard the staining solution.
12. Wash several times with 80% ethanol until the washing solution becomes clear.
13. Wash in water for 20 sec with gentle agitation.
14. Cover with 4% buffered paraformaldehyde and store up to 2 weeks at room temperature.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Anesthetic mixture

0.75 ml xylazine (Rompun, Bayer)
1 ml ketamine (Imalgene 1000, Merial, Virbac)
4 ml distilled water
Store for no longer than 1 month at 4°C

Buffered paraformaldehyde (PFA) fixative, 4% (w/v)

Stock solution (20% PFA): Add 20 g paraformaldehyde (e.g., Merck) to 80 ml of 1× PBS (e.g., Sigma-Aldrich). Mix well and fill up to 100 ml with 1× PBS. Heat at a maximum of 65°C in a conical flask, while stirring vigorously on a magnetic stirrer, until the solution contains no visible paraformaldehyde aggregates. Allow to cool and store in 10-ml aliquots up to 6 months at –20°C.

Working solution (4%): Thaw stock solution and dilute 1 vol with 4 vol of PBS in a conical flask (do not refreeze remaining stock solution). Mix well and filter through filter paper to remove any precipitate. Cool to ∼4°C on crushed ice. Adjust pH, if necessary, to pH ∼7.4 with 1 M NaOH. Prepare immediately before use.

En face staining solution containing Sudan IV

250 ml acetone
175 ml 100% ethanol
2.5 g Sudan IV (e.g., Sigma-Aldrich)
Water to 500 ml
Filter with filter paper and store up to 6 months at room temperature in a dark bottle

Eosin Y, 0.1% (w/v)

Dissolve 1 g eosin Y (e.g., Bayer) in 1 liter deionized water. Add ∼4 drops of HCl to adjust pH in the range of 4 to 5. Store at room temperature (stable at least 2 months).

Oil red O staining solution

Saturated stock solution: Add 500 mg oil red O powder (e.g., Sigma) to 100 ml of 99% isopropanol and mix. Store up to 6 months at room temperature.

Working solution: Dilute 30 ml saturated stock solution in 20 ml deionized water and vortex. Allow to stand 10 to 15 min at room temperature, and filter through a syringe-driven filter unit (0.22-μm pore size, e.g., MillexGP). The filtrate can be used for several hours.

Picro-Sirius red staining solution, 0.1% (w/v)

Prepare a saturated, aqueous picric acid solution by dissolving 7.5 g moist solid picric acid (35% H_2O; e.g., Sigma-Aldrich) in 500 ml deionized water (some precipitates must remain in the flask). Dissolve 0.5 g Sirius red F3B (Direct red 80, Avocado) in 500 ml saturated, aqueous picric acid solution. Filter through filter paper (e.g., Rapid flow filter paper, Macherey-Nagel) before use. The solution is stable for at least 3 years at room temperature and can be reused several times. After each use, add a little solid picric acid to ensure saturation.

CAUTION: *See Basic Protocol 2 for cautionary note on picric acid.*

Schiff's reagent (Gabe, 1968)

Add 13 ml of 37% HCl to 1 liter deionized water. Dissolve first 5 g $Na_2S_2O_5$ and then 5 g pararosaniline in 1 liter of the 0.15 M HCl. Mix slowly on a magnetic stirrer for 2 hr at room temperature. Add ~3 g fresh activated charcoal, stir vigorously for 2 min, and filter. The solution should be clear. If it shows a pink or yellow color, repeat the charcoal decolorization using a fresh lot of activated charcoal. Store at 4°C and away from light in ultra-clean 500-ml bottles wrapped in aluminum foil. When the reagent becomes pink, it is no longer usable. Discard Schiff's reagent after each use, i.e., do not reuse.

Silicone-coated Petri dishes

Using reagents provided in the silicone elastomer kit (World Precision Instruments), mix 10 parts base (silicon elastomer) with 1 part curing agent prior to use. Place at 4°C and mix slowly to avoid bubble formation. Slowly pour 20 ml into 90-mm Petri dishes. Leave for a few hours at 4°C to eliminate all air bubbles, then leave overnight at room temperature. Dry dishes 24 hr in a 60°C oven.

Succinate dehydrogenase staining solution

5 ml 0.2 M KH_2PO_4
19 ml 0.2 M Na_2HPO_4
25 ml 0.2 M sodium succinate solution
1 ml 10% (w/v) nitroblue tetrazolium (NBT) solution
Adjust pH to 7.35 with 1 M NaOH
Prepare immediately before use

COMMENTARY

Background Information

Necropsies and histopathological analysis are highly technical procedures that can yield a great deal of information. In addition to technical skills, an important amount of prior knowledge is required for morphological analyses. The investigator must be familiar with characteristics of human and mouse anatomy and human and mouse histology. For this type of background information, the reader is referred to Williams et al. (1989) for human anatomy; Krstic (1984), Fawcett (1994), and Sternberg (1997) for human histology; Popesko et al. (1992) for mouse anatomy; and Maronpot et al. (1999) and Mohr et al. (1996) for mouse histoloy. Histological protocols usually include the following steps: fixation, tissue processing, embedding, and staining (McManus and Mowry, 1960; Lillie, 1965; Gabe, 1968; Ganter and Jollès, 1970; Luna, 1992; Carson, 1997; also see Internet Resources for Histosearch and StainsFile).

Fixation

To minimize post-mortem changes in cell structure, fixation should be accomplished very quickly. Optimal tissue preservation requires delivering the fixative as close to each cell as possible. This can be accomplished by immersion or perfusion. When an organ is immersed in a fixative, its periphery is fixed more rapidly than central areas, which are reached by the fixative with some delay and may undergo postmortem alterations.

Moreover, and unrelated to the problem of postmortem changes, there is a gradient in the quality of fixation in immersion-fixed tissues, which may yield experimental artifacts (e.g., uneven shrinkage, hardening) leading to false interpretations. Since ideally all cells should be exposed simultaneously to the same concentration of fixative, successful fixation by immersion requires the use of small samples. Despite the inevitable gradient of fixation quality in immersion-fixed organs, there is a minimum size required for the tissue sample, since there is a risk of introducing mechanical damage at the surface layers of the sample. Perfusion fixation, which involves the delivery of fixative through the circulatory system of living tissue by direct injection into the heart or a major artery, represents the quickest way to fix tissues and organs.

Tissue processing

Once stabilized and hardened by fixation, tissue specimens are embedded in a solid medium, which will support the production of thin tissue slices destined for microscopic examination. The paraffin embedding method is the most suitable for routine preparation, sectioning, and storage of large numbers of tissue samples. A fixed tissue cannot, however, be directly embedded in paraffin; its free water must first be replaced by solvents. The technique of getting the fixed tissue into paraffin (or other embedding medium) is commonly referred to as tissue processing. The three steps in this process are dehydration, clearing, and paraffin infiltration.

Dehydration is usually done with a series of alcohols, typically 70%, 95%, and 100% ethanol. There is no benefit to slower stepwise dehydrations. It is important that the first alcoholic solution following the 10% neutral buffered formalin should contain no more than 70% alcohol to prevent precipitation of the buffer salts. If water is left in the tissue as a result of improper dehydration, the clearing agent and thus the paraffin will not penetrate the tissue, which will remain soft and be impossible to cut. In contrast, excessive dehydration will remove bound water, yielding shrunken, hard, and brittle specimens.

The clearing step consists of replacing the dehydrant with a substance that is miscible with the embedding medium. For paraffin, a common clearing agent is xylene. Newer clearing agents that present a reduced health hazard are based on limolene or aliphatic long-chain hydrocarbons.

Finally, the tissue is infiltrated with the embedding paraffin, which is subsequently solidified by cooling. Paraffin is an inert mixture of hydrocarbons, and can contain plastics, beeswax, or other additives. Commercial paraffins differ in melting temperature for different hardnesses. Paraffin with a melting point of 56° to 58°C is commonly used for routine histology. A vacuum can be applied inside the tissue processor to assist penetration of paraffin.

Sectioning

Sectioning is the production of thin slices from a tissue sample. Tissues embedded in paraffin can be sectioned anywhere from 3 to 15 μm thick, with 5 μm being typical for routine use. During the embedding process, the tissues must be oriented properly in the block of paraffin to generate transverse or longitudinal sections. Once the tissues have been embedded, they are cut into sections that are collected on glass slides for subsequent histological, histochemical, or immunohistochemical staining. Sectioning is done with a microtome, which is nothing more than a very sharp knife attached to a mechanism for advancing the paraffin block at standard distances. Once sections are cut, they are floated on warm water to remove any wrinkles, and then transferred to glass slides. The glass slides are then dried in a warm oven to help the sections adhere to the slide. The use of an adhesive mixture such as a gelatin solution enhances the adherence.

Staining

Most cells contain no endogenous pigments and are thus transparent. Stains are used to establish contrast to render tissue components visible. Prior to staining, the embedding process must be reversed to remove paraffin from the tissue and allow water-soluble dyes to penetrate the histological section. Slides are deparaffinized by running through solvents (e.g., xylene), graded alcohols, and then water.

Routine histology uses the stain combination of hematoxylin and eosin, which allows for visualization of cellular structures. Other stains bind selectively to particular tissue components and are used to identify specific structures. It is noteworthy that all stain color is artifactual (i.e., it does not represent the natural color of the tissue). A given structure may have very different colors with different stains. For example, collagen is pink with hematoxylin and eosin, but blue or green with trichrome stains, and red with the Sirius red stain.

Therefore, specific aspects of actual structure (location, size, shape) should be used to identify cells and tissues rather than color, which is unreliable. It should also be stressed that a histological section is to be regarded as a single snapshot of the life of the organ. Information regarding the dynamics of a biological process (cell movement, direction of cell proliferation, or cell fate) cannot be deduced solely on the basis of structural patterns.

Critical Parameters

Fixation

Formalin. Commercial solutions of concentrated formalin sometimes become turbid during storage through the production of paraformaldehyde, which decreases their strength. For the sake of standardization, a turbid solution should be discarded, and any opened bottle of concentrated formalin should be used within 6 months. Formaldehyde has a greater chance for oxidation at low concentrations, and eventually the pH of a 10% neutral buffered formalin solution will start to decrease in spite of the buffer. In addition, 10% buffered formalin solutions will have slowly increasing concentrations of formic acid upon storage (an unwanted byproduct of aging formaldehyde). This promotes the clumping of proteins instead of the cross-linking that formaldehyde normally promotes. Therefore, if the tissue is destined for antibody staining at a later time, 10% buffered formalin solutions should be used within 3 months (McManus and Mowry, 1960; also see Internet Resources). A loss of antigenicity during prolonged formalin fixation is caused both by extensive cross-linking of proteins (preventing access of the antibody to the antigen) and by chemical alteration of the antigen (i.e., change in epitope structure). However, antigens can often survive a short exposure to formalin. On the other hand, the duration of fixation must be long enough to preserve the integrity of the tissue and prevent antigen diffusion. This is a dilemma known as the "immunohistochemical compromise". Although tissues may be stored in buffered formalin indefinitely without morphological damage, they should not be fixed for more than 24 hr if immunohistochemistry will be performed.

Bouin's solution. Due to deleterious effects on tissue morphology of prolonged exposure to the acids present in Bouin's solution, it is recommended not to keep specimens in this fixative for more than 1 month before paraffin embedding.

Buffered glutaraldehyde. As a general rule, the quality of this fixation depends on the quality of the perfusion. Organs are kept in the fixative at 4°C. Samples destined for electron microscopy should never be stored in ethanol, as alcohol will extract lipids from the tissues.

Buffered paraformaldehyde. The duration of fixation should be minimal to prevent loss of antigenicity caused by extensive cross-linking of proteins (preventing access of the antibody to the antigen) and chemical alteration of the antigen (i.e., change in epitope structure). On the other hand, the duration of fixation should be long enough to preserve the integrity of the tissue and prevent antigen diffusion (the so-called immunohistochemical compromise).

Perfusion. Perfusion speeds up the delivery of the fixative, but does not affect its chemical action on the tissue. For this reason, perfusion fixation is always followed by immersion fixation with the same fixative for a few hours.

Freezing. Slow freezing generates large ice crystals that cause holes in tissues. For this reason, freezing dissected tissues in a cryostat, in a freezer, or on dry ice is not recommended. On the other hand, snap-freezing samples by dipping them directly into liquid nitrogen generates a violent shock that can yield large breaks in the tissues. Thus, special freezing conditions (i.e., in liquid nitrogen vapors, in cold isopentane) need to be used.

Staining

Hematoxylin and eosin. Do not allow the sections to dry at any point during the staining procedure. Within 12 hr before use, the hematoxylin solution should be filtered to remove metallic precipitants that form during storage. Decreased hematoxylin staining, evaluated by microscopic inspection of the sections, indicates that the staining solution has been overused and should be discarded. This occurs after ~500 slides. Decreased eosin staining indicates that the staining solution has aged or has been overused and should be discarded.

Oil red O for neutral fats. In some tissues (e.g., liver), neutral lipid content can change within a few hours of fasting. It should also be noted that the intracellular localization of lipid stores may be obscured by some amount of displacement that occurs during cryostat sectioning, staining, and mounting. A fresh frozen section of ovary can be used as a positive control.

Osmium tetroxide for unsaturated lipids. Osmium tetroxide has a low penetrating power. The tissue must be cut into small blocks prior to fixation.

Immunodetection of lipid droplet ghosts with anti-adipophilin antibodies. The commercial antibody solution should be stored in 10-µl aliquots at −80°C. Once an aliquot has been thawed, it should not be refrozen. Working dilutions of antibodies should be used within 3 days and kept at 4°C.

Staining for cholesterol esters. Sections, which are mounted in concentrated sulfuric acid, should be handled with great care and examined within 5 min. The presence of cholesterol esters in the tissue should be confirmed using thin layer chromatography of lipid extracts from the tissue (see Mascrez et al., 2004, for details).

Periodic acid Schiff (PAS) for glycogen. Use only ultra-clean glassware rinsed with deionized water for making, storing, and staining with the Schiff's reagent. Each experiment should include a positive control such as a section of the duodenum to test the efficacy of the stain, and a negative control to validate the specificity of the PAS stain for glycogen. A suitable negative control is obtained by incubating a liver section for 10 min at room temperature with a 10 mg/ml solution of alpha amylase from human saliva (Sigma) in 1× PBS (Carson, 1997).

Sirius red for collagen fibers. It is necessary to rotate the polarizing filters to see all the collagen fibers, because in any single orientation, the birefringence of some fibers will be masked.

Succinate dehydrogenase for mitochondria. Muscles containing both oxidative and glycolytic fibers display a heterogeneous pattern of SDH activity. It follows that equivalent regions of the same muscle should be compared between mice to avoid misinterpretations.

En face staining of aortas with Sudan IV. The percentage of the surface covered by plaques (red areas) should be quantified by computer-assisted analysis.

Anticipated Results

Examples of fixation methods and examples of hematoxylin and eosin staining on various tissues are presented in Figure 29B.4.4. This figure also provides examples of first-line extended assays. Similarly, examples of different specialized stains are illustrated in Figure 29B.4.5.

Fixation

Formalin. A 10% (v/v) formalin solution in a neutral phosphate buffer is the most commonly used fixative for routine histological analyses of adult tissues, as it penetrates the tissues rapidly and can be used for long-term storage.

Bouin's solution. Bouin's solution, a mixture of formalin, picric acid, and acetic acid, is the fixative used routinely for mouse embryos, fetuses, placentas, as well as adult testes. It preserves nuclear details and chromosomes better than buffered formalin, and avoids distortion of extracellular spaces introduced upon formalin fixation in some tissues such as the seminiferous epithelium of the testis.

Buffered glutaraldehyde. Buffered glutaraldehyde (2.5% v/v) is a widely used fixative for the production of semi-thin, resin-embedded sections for light microscopy and of thin sections for electron microscopy.

Buffered paraformaldehyde. A 4% (w/v) solution of buffered paraformaldehyde, which is nothing else but freshly prepared formalin, is the most commonly used fixative for immunohistochemistry and in situ hybridization analyses.

Perfusion. Perfusion represents the quickest way to fix tissues, thereby allowing optimal preservation of cellular sub-structures as well as of mRNAs and antigens. It is recommended for samples destined for immunohistochemical, in situ hybridization, and electron microscopic analyses. Because it is time consuming, it is not employed for routine histology.

Freezing. Freezing provides an alternative to chemical fixation and is followed by direct sectioning of the frozen specimen. Frozen sections are not used in routine histology because they lack histological detail.

Staining

Hematoxylin and eosin. Nuclei and nucleoli are purple, and the chromatin should be very distinct. Correct hematoxylin staining shows crisp staining of the nuclear chromatin, demonstrating well-delineated nuclear contours and sharply stained heterochromatin against weakly stained euchromatin. Cytoplasm, muscles, collagen fibers, and erythrocytes appear in varying shades of pink. Mucins and cartilages are light blue.

Oil red O for neutral fats. Neutral lipids (mainly triglycerides and cholesterol esters) in liquid phase are bright red. Nuclei are typically counterstained, either brightly fluorescent (with DAPI) or purple (with hematoxylin).

Osmium tetroxide for unsaturated lipids. Unsaturated lipids are brown or black. Osmium tetroxide allows for the localization of

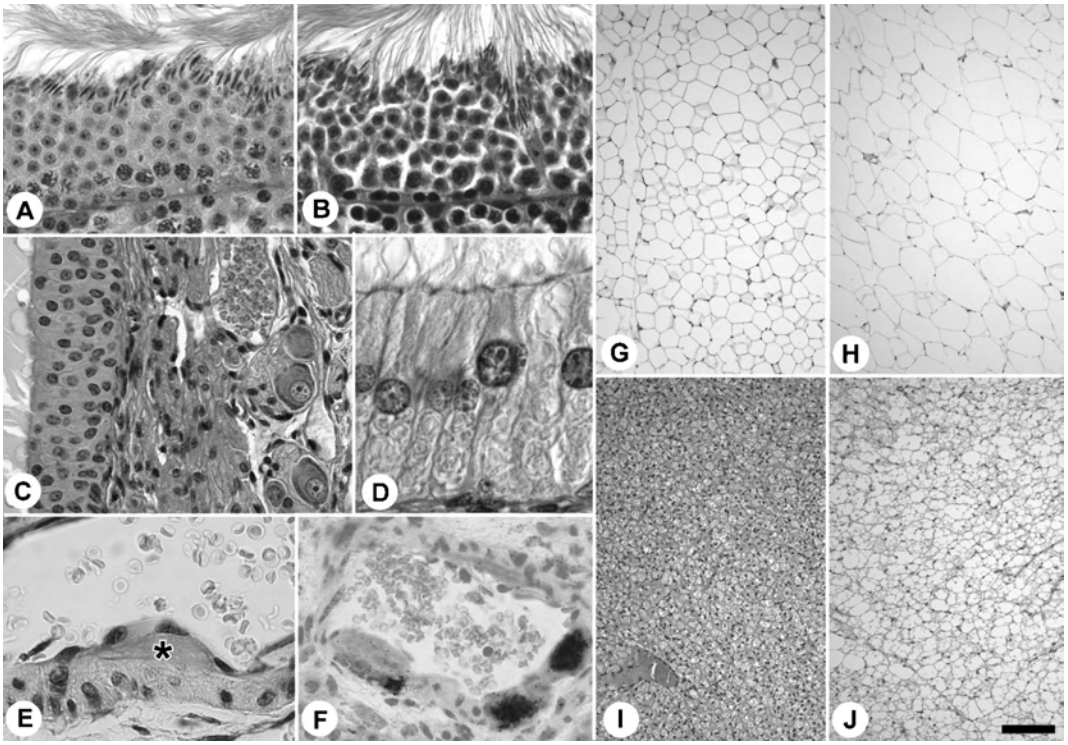

Figure 29B.4.4 Applications and limits of routine buffered formalin fixation and hematoxylin and eosin staining. A solution of 10% neutral buffered formalin preserves most tissues appropriately. The testis presents an exception: its seminiferous epithelium (here stained with hematoxylin and eosin) appears much more "life-like" after Bouin's fixation (**A**) than after formalin fixation (**B**). In contrast, the urinary bladder (**C**, showing from left to right lumen, epithelium, lamina propria, and parasympathetic neurons), the epithelial cells of the vas deferens (**D**), the small kidney artery (**E**), white adipose tissue (**G,H**), and brown adipose tissue (**I,J**), all stained by hematoxylin and eosin, are not distorted upon buffered formalin fixation. Hematoxylin and eosin allows for the distinction of nuclei from cytoplasm (e.g., in **D**) and of cells from extracellular matrices (asterisk in **E**). In some tissues, hematoxylin and eosin staining defines cell contours and thus allows measurement of cell surfaces; examples are shown for white fat adipocytes (**G,H**) and brown fat adipocytes (**I,J**) in wild-type (**G,I**) and mutant (**H,J**) mice fed a normal diet. Specialized stains can provide additional information. In small kidney arteries of a mutant mouse, the hematoxylin and eosin stain has allowed detection of abnormal subendothelial deposits (asterisk in **E**). A cross-section through one of the mutant arteries (**F**), stained with oil red O and the von Kossa stain for calcium (not covered in this unit), shows that the abnormal deposits contain lipofuscins (red) and are calcified (black). Scale bar: 40 μm (**A-C,F**), 15 μm (**D,E**), and 320 μm (**G-J**). For color version of this figure, see *http://www.currentprotocols.com.*

the lipid inclusion at the cellular level, whereas oil red O allows localization only at the tissue level in most cases.

Immunodetection of lipid droplets with anti-adipophilin. The envelopes of lipid droplets appear as bright red fluorescent vesicles or dots, whereas nuclei display a blue fluorescence. The interstitial layer of the ovary and the stellate cells of the liver provide excellent positive controls for adipophilin immunostaining.

Staining for cholesterol esters. Cholesterol esters are violet. Any brown staining is unspecific. Tissues that are rich in lipids (i.e., stain positively with oil red O) should be used as positive (e.g., adrenal gland) and negative (e.g., interscapular brown adipose tissue) controls for cholesterol esters.

Periodic acid Schiff (PAS) for glycogen. Glycogen in hepatocytes, mucins of intestinal tract, and acrosomes of spermatid stain magenta or purple, and basement membranes stain magenta. These structures are said to be PAS-positive. In contrast, collagen is PAS-negative.

Sirius red for collagen fibers. In bright-field microscopy, collagen is red on a pale yellow background. When examined through cross-polarized filters, larger collagen fibers are bright yellow or orange, and thinner ones are green. According to Junqueira et al. (1979), the birefringence is highly specific for fibrillar

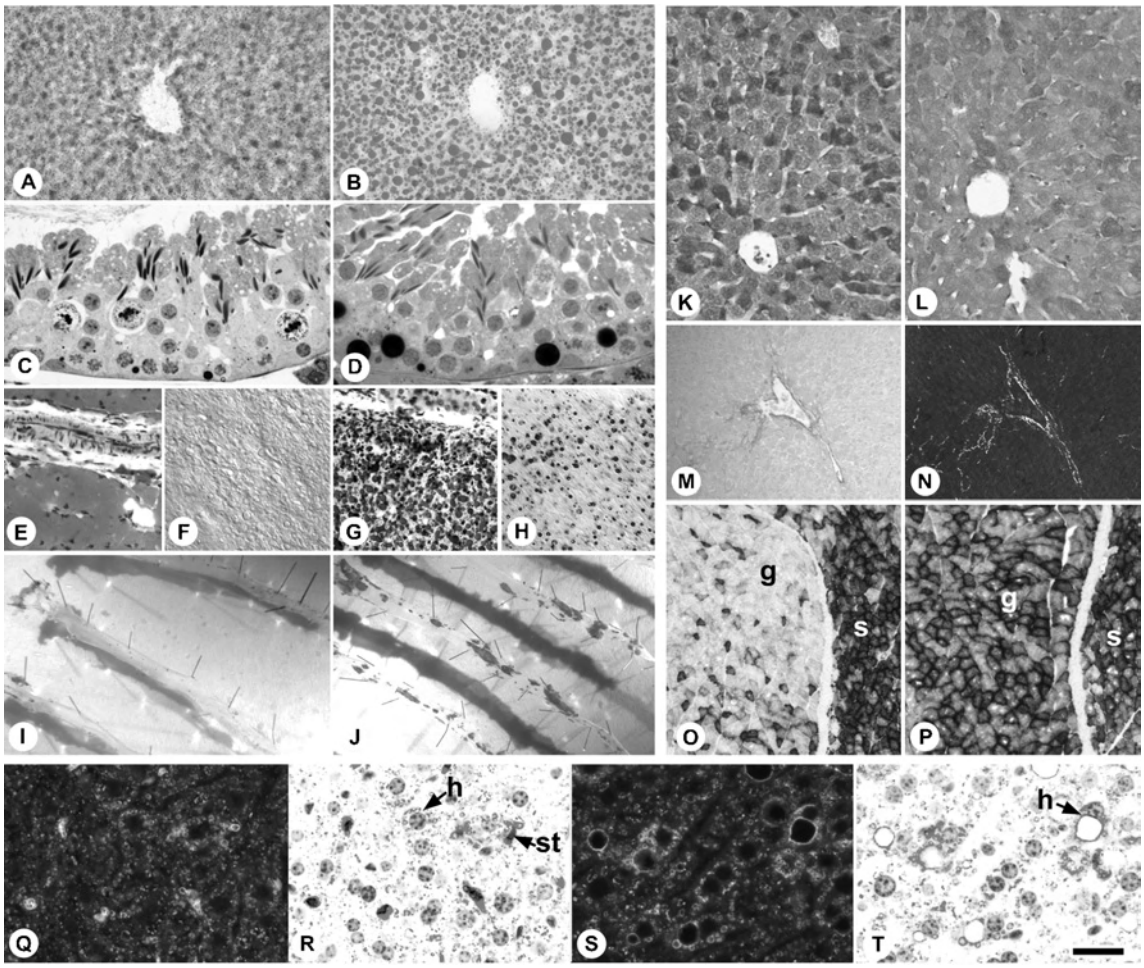

Figure 29B.4.5 Special staining techniques in metabolic studies. (**A,B**) Oil red O staining in liver from wild-type mice fed a normal chow (**A**) or high-fat (**B**) diet. Blue spots correspond to computer-generated negative images of a DAPI nuclear counterstain. (**C,D**) Osmium tetroxide staining in seminiferous tubules of wild-type and mutant testis, respectively. Note the increase in the size of the fat droplets (black dots) in the mutant seminiferous epithelium. The nuclear counterstain is toluidine blue. (**E**-**H**) Detection of cholesterol esters. Both brown adipose tissue (BAT) and adrenal cortex of wild-type mice contain large amounts of neutral fats (bright red, **E** and **G**), but cholesterol esters are only detected in the latter tissue (compare **F** and **H**). BAT and adrenal cortex provide excellent negative and positive controls in this detection procedure. (**I,J**) En face staining of aortas. Bright red patches indicate atherosclerotic lesions in younger (**I**) and older (**J**) mutant mice. (**K,L**) The periodic acid Schiff (PAS) reaction shows that the glycogen normally present in wild-type hepatocytes (magenta stain in **K**) is almost undetectable in the liver of a mutant mouse (**L**). (**M,N**) Sirius red staining of pathological collagen deposits in the liver, after carbon tetrachloride administration, can be observed under normal (**M**) and polarized (**N**) light. (**O,P**) Succinate dehydrogenase activity illustrates the difference in the mitochondrial content of gastrocnemius muscle fiber (g) between wild-type (**O**) and mutant (**P**) mice (s, soleus). (**Q**-**T**) Immunodetection using an anti-adipophilin antibody labels the contours of lipid droplets in hepatocytes (h) and in stellate cells (st) of the liver. The increase in the size of lipid inclusions in the hepatocytes of a mouse fed a high-fat diet (**S,T**) is conspicuous, compared to a mouse fed a normal chow diet (**Q,R**). (**A,B,E**-**H,O,P**) Frozen sections. (**C,D**) Sections from resin-embedded tissues. (**K**-**N,Q**-**T**) Sections from formalin-fixed, paraffin-embedded tissues. Scale bar: 160 μm (**A,B,E**-**H**), 40 μm (**C,D,Q**-**T**), and 80 μm (**K**-**P**). For color version of this figure, see *http://www.currentprotocols.com.*

collagen. A few other materials are stained red but are not birefringent. Portal tracts of normal livers can be used as positive controls.

Succinate dehydrogenase for mitochondria. The histochemical reaction results in a blue speckled pattern of the mitochondria, proportional to the number of mitochondria and the SDH activity within them. The more SDH (and therefore mitochondria) a cell contains, the greater the intensity of the stain. A transverse section of a muscle should be used as a positive control: oxidative fibers have

a dense purple speckled appearance, while nonoxidative fibers have only scattered purple speckles.

En face staining of aortas with Sudan IV. Neutral lipids in atherosclerotic plaques are bright red.

Time Considerations

Pre-existing knowledge of tissue location and practice in tissue dissection and handling are indispensable prior to necropsy to ensure tissue collection in the minimum amount of time. A complete necropsy requires ~45 min per mouse. Collecting tissue for RNA analyses should take no longer than 1 to 10 min, depending on the organ. If RNA sampling is desired, the dissections should be performed by two persons—one to dissect the tissues destined for RNA analysis and freeze them in liquid nitrogen, and the other to collect tissues for histological analyses.

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Internet Resources

http://www.histosearch.com

Histosearch is a search engine that searches over 20,000 Web pages from histology-related sites.

http://home.primus.com.au/royellis/fix.htm

Informational site for fixation and fixatives by A.S.-Y. Leong.

http://stainsfile.info/StainsFile/jindex.html

StainsFile is a useful site with general information and resources for histology.

http://swehsc.pharmacy.arizona.edu/exppath/resources/formaldehyde.html

Information on formaldehyde fixatives at the Southwest Environmental Health Sciences Center (SWEHSC), University of Arizona College of Pharmacy.

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Dietary Manipulation of Mouse Metabolism

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ABSTRACT

The maintenance of metabolic homeostasis relies on the balanced intake of nutrients from food. Consequently, diet composition strongly impacts whole-body physiology. Dietary formulations with strong nutrient imbalances can lead to metabolic disorders, with lipids and simple sugars playing a prominent role. This unit describes how diet formulation can be modified to generate mouse models of human metabolic pathologies, and it details methodological procedures linked to dietary manipulations, including caloric restriction and introduction of a test compound. *Curr. Protoc. Mol. Biol.* 84:29B.5.1-29B.5.12. © 2008 by John Wiley & Sons, Inc.

Keywords: metabolism • diet • mouse • diabetes • obesity

INTRODUCTION

Nutrition strongly modulates risk factors in the development of metabolic disorders and chronic diseases, including obesity, metabolic syndrome, type 2 diabetes, and cardiovascular diseases. In order to gain a greater understanding of human metabolic disorders, mice are commonly used models because they will readily develop these pathologies when provided with an appropriate diet. Basic Protocol 1 presents the nutritional composition guidelines of a standard chow diet and of specialized diets commonly used to induce metabolic disorders. This is followed by protocols for administering a test compound through the diet (Basic Protocol 2) and performing a caloric restriction experiment (Basic Protocol 3).

INDUCTION OF METABOLIC PATHOLOGIES VIA DIETARY MANIPULATION

BASIC PROTOCOL 1

Standard chow diet is formulated to closely match the daily caloric requirements of mice (10 to 15 kcal/day) when provided ad libitum and to respect the animals' nutritional needs. Chow diets are generally formulated by commercial animal food suppliers, to which the reader is referred for a detailed composition. Because these diets are manufactured mainly from agricultural products, their composition is not tightly defined. Nevertheless, the main components include cereals as the major source of carbohydrates and fiber (~85% by weight), soy and yeast extracts as sources of vegetable proteins (~8% by weight), fish extracts as sources of animal protein and fatty acids (~3% by weight), ash for mineral supply (~4% by weight), and traces of mineral and vitamin complements formulated to match the animals' metabolic needs. The total fat content is composed of roughly equivalent amounts of saturated and unsaturated fatty acids, with palmitic, oleic, and linoleic acids being the most abundant lipids. The caloric intake from standard chow is ~2900 kcal/kg; the approximate energetic composition is specified below as percent of the total energy content:

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Fat ~5% to 12%
Proteins ~20% to 25%
Carbohydrates ~65% to 70% (~65% starch and 2% to 3% simple carbohydrates).

As summarized in Table 29B.5.1, diet modifications can be used to induce a variety of pathologic conditions in laboratory mice. These studies are typically carried out in the C57BL/6 background, which is relatively susceptible to dietary manipulation, or in genetically manipulated animals in the C57BL/6 background. If other strains or animals on a mixed background are used, it is essential to include appropriately matched control animals, restricted to wild-type littermates from a heterozygous breeding for animals on a mixed background.

In a typical experiment, animals of different genotypes or in different experimental conditions are challenged with one or more experimental diets, and their biological responses under these diets are compared to those of animals on a control chow. While it is not possible to outline a single protocol to cover all such experiments, key parameters to consider include using appropriate numbers of age- and gender-matched mice (see Critical Parameters for more details) and maintaining the diets for sufficient times to induce the desired responses. Typical times required for the induction of various pathologies are indicated in Table 29B.5.1.

Most of the diets described in Table 29B.5.1 are generally formulated by commercial animal food suppliers. Alternatively, experimenters with specific requirements may want to have custom diets formulated either by commercial food suppliers that offer this service or by academic facilities that specialize in animal nutrition. In any case, it should be kept in mind that most of these diets are only partially defined diets, and variations in the composition of certain constituents can modify the biological response in vivo. For example, a high-fat diet is most commonly made from animal fat using lard, but the nature of lard triglycerides can vary according to the specific origin and can thereby affect the biological outcome.

BASIC PROTOCOL 2

ADMINISTRATION OF A TEST COMPOUND THROUGH THE DIET

Incorporating a compound in the diet to test for its physiological actions is an easy and efficient way to achieve reproducible daily exposure with minimal stress to the animals. The type of diet in which the compound is mixed should be chosen, on the one hand, according the appropriate diet for the purpose of the study and, on the other hand, according to the chemical properties and bioavailability of the compound itself. Indeed, the polarity of the compound can influence its bioavailability. For example, in the case of a lipophilic molecule, the fat content of the diet can modify its intestinal absorption by increasing its incorporation into lipid micelles. In addition, technical considerations relating to the amount of compound to incorporate, its polarity, and the type of diet chosen should be evaluated in order to achieve a homogeneous incorporation, which will ensure constant and homogeneous dosing of the animals (see Critical Parameters and Fig. 29B.5.3).

Materials

Appropriate mouse strain (e.g., C57BL/6 or see Commentary)
Mouse diet (standard or high-fat diet) in powdered form
Test compound
Vehicle to dissolve compound (if required)

Group housing cages for mice
Scale with 0.1 g accuracy (to weigh food and mice)
Container to accommodate mice during weighing
Scale with 0.1 mg accuracy (to weigh compound)

Table 29B.5.1 Mouse Diets Commonly Used to Induce Metabolic Disorders

Induced pathology	Type of diet	Composition guidelines[a]	Appropriate mouse strain	Duration to develop pathology[b]	Reference
Obesity	High fat	Fat ~40%[c]	C57BL/6	8-12 weeks	West et al., 1992; Surwit et al., 1995
	Very high fat	Fat ~60%[c]		8-12 weeks	Surwit et al., 1995; Rossmeisl et al., 2003
Hypertriglyceridemia + insulin resistance (IR)	High sucrose diet	Sucrose ~50%-60%	Mice develop sucrose-induced IR very slowly	30-55 weeks	Surwit et al., 1995; Sumiyoshi et al., 2006
Metabolic syndrome (obesity + IR)	High fat/high sucrose	Fat ~40% Sucrose ~30%[d]	C57BL/6	14-18 weeks	Surwit et al., 1995; Parekh et al., 1998
Ketoacidosis	Very high fat, low carbohydrate	Fat ~84% Carbohydrate 0%	C57BL/6	4-8 weeks	Kennedy et al., 2007
	High fat/high protein, low carbohydrate	Fat ~65%/protein ~35% Carbohydrate ~0%	C57BL/6	4-8 weeks	
Hypercholesterolemia + atherosclerosis	High fat/high cholesterol + cholic acid	Fat ~15% Cholesterol ~1.25% Cholic acid ~0.25%-0.5%	C57BL/6	14-18 weeks	Paigen et al., 1985, 1987
	High cholesterol	~1.25%	LDLR KO Apo E KO	8-12 weeks	Getz and Reardon, 2006; Hartvigsen et al., 2007
Hypertension	High salt	NaCl ~8%	Mice develop salt-induced hypertension slowly	12-16 week	Yu et al., 2004
	High salt + DOCA + nephrectomy[e]	NaCl ~8% DOCA ~3.5 mg/kg/day	C57BL/6	2-4 weeks	Hartner et al., 2003; Monassier et al., 2006

[a]Composition guidelines are only given when differing from chow diet and are expressed in percentage of the total energetic content, except for NaCl which is a percentage of weight.

[b]The duration for inducing the pathologies described can vary according to the age at which the dietary intervention is started, with induction times being reduced in aged animals. In addition, disease progression will evolve gradually, and the importance of the pathologies developed will therefore be directly correlated to the length of the dietary intervention.

[c]It should be noted that high-fat and very-high-fat diets generally also have elevated sucrose content (from 7% to 20%), which also contribute to the induction of obesity. In addition, obesity and insulin resistance are tightly linked, and such diets will also promote insulin resistance, although the phenotype on glucose homeostasis will generally appear after the first signs of obesity.

[d]Fructose is also efficient at inducing insulin resistance and can replace sucrose.

[e]Deoxycorticosterone acetate (DOCA) is most generally administered subcutaneously by daily injection or by implantation of slow-releasing pellets, and NaCl can also be administered through drinking water at a concentration of 1% (w/w). In addition, DOCA-salt hypertension is maximized by unilateral nephrectomy.

Mortar and pestle for grinding the compound (if required)
Large container for mixing diet and test compound *or* automated mixing machine
Commercial pelleting machine (optional)
Desiccator (optional)

Determine daily food intake

The accuracy of the doses administered through food relies on the correct measurement of food intake (FI), which directly controls the amount of compound ingested. Therefore, FI should be evaluated, always housing the same number of animals per group.

1. At a given hour of the day, change mice to clean cages and weigh the amount of food given.
2. Two to seven days later, at the same hour of the day, weigh the food remaining, including any chunks of pellets in the cage.
3. Calculate FI in g/mouse/day according to the formula:

$$\mathrm{FI}\left(\mathrm{g/mouse/day}\right)=\frac{w_0-w_{n\mathrm{d}}}{n_{\mathrm{d}}\times n_{\mathrm{m}}}$$

Equation 29B.5.1

where w_0 is the weight (g) of the amount of food given;

$w_{n\mathrm{d}}$ is weight (g) of the food remaining in the cage at the end of the feeding period;

n_{d} is the number of days over which FI is calculated;

n_{m} is the number of mice per cage.

4. Repeat the measurement every two days during the first week of treatment to verify that the compound does not cause food aversion, and on a weekly basis thereafter.

Calculate test compound dose

A test compound is generally administered at a constant dose normalized to body weight (mg compound/kg body weight). Since accomplishing this for each individual animal would require individual housing and preparation of a diet with a concentration of compound adapted to each mouse, a widely accepted simpler approach consists of using the average body weight (BW) and food intake (FI) as an estimate for each animal. Alternatively, in the experimental case where both the diet and the treatment are expected to minimally affect body weight, the compound can be administered at a constant concentration in the diet. The concentration should be calculated the same as for a constant dose, but only once, using the BW and FI calculated before the beginning of the treatment or from estimates of these parameters derived from pilot studies performed in the exact same experimental setting.

5a. *To calculate test compound as a constant dose in diet (mg/kg body weight)*: Adjust the quantity of compound to incorporate into the diet (QCD) given on a weekly basis by calculating for week n the QCD_n in mg compound/g diet according to the following equation:

$$\mathrm{QCD}_n=\frac{\mathrm{LE}\times\mathrm{BW}_{n-1}}{\mathrm{FI}_{n-1}}$$

Equation 29B.5.2

where LE is the desired level of exposure (mg/kg BW/day);

BW_{n-1} is the average body weight (kg) from week the previous week (n-1);

FI_{n-1} is the food intake (g/mouse/day) from week the previous week (n-1).

The incorporation of the compound into the diet requires weekly adaptation to adjust for variations in FI and BW. Thus, these parameters should be recorded on at least a weekly basis, at a controlled time of day, as described in steps 1 to 4.

5b. *To calculate test compound as a constant concentration (mg/kg diet):* Calculate the concentration of test compound to be incorporated into the diet, using Equation 29B.5.2 and the BW and FI calculated before the beginning of the treatment or from estimates of these parameters derived from pilot studies performed in the exact same experimental setting.

After validating the stability (in the diet) of the compound to be incorporated, this alternative has the advantage of allowing the preparation of a large stock of pellets for long treatment periods, and therefore it has the potential to outsource the pelleting process.

Prepare pellets containing test compound

6a. *To incorporate a compound into a high-fat diet:* Mix the compound directly with the high-fat diet powder, using either manual mixing in a large container or an automated mixing machine.

The high lipid content of high-fat diet enables direct compaction.

Incorporating low amounts (<100 mg/kg) of compound will require using an appropriate vehicle to ensure homogeneous incorporation (see Critical Parameters and Fig. 29B.5.3 for details).

6b. *To incorporate a compound into a standard diet:* Incorporate 600 ml/kg autoclaved distilled water in standard chow diet powder. Mix in the powdered compound, using either manual mixing in a large container or an automated mixing machine.

Adding the water enables compaction.

Incorporating low amounts (<100 mg/kg) of compound will require using an appropriate vehicle to ensure homogeneous incorporation (see Critical Parameters and Fig. 29B.5.3 for details).

7. Form pellets manually (by tightly packing the mixture) or using a commercial pelleting machine (recommended).

Alternately, animals can be fed the powdered diet (see step 9 annotation).

8. Store high-fat diet pellets up to 2 months at 4°C (or frozen according to the manufacturer's instruction) until use. Air-dry chow diet pellets in a desiccator or in a ventilated hood for at least 48 hr and store up to 6 months at room temperature or according to the stability of the compound (see Critical Parameters).

Administer test compound in diet

9. Give a large excess of pellets (at least 20% more than the amount required according to FI on week n-1) in a clean cage, at a weekly frequency (minimum).

Although animals can be fed the powdered diet from step 1, it is essential that specialized equipment be used, as mice will spill large quantities of food and rapidly contaminate open troughs. For example, special troughs with grids have been designed to limit these problems, and one is illustrated in Figure 29B.5.1. To avoid these problems entirely, it is best to restrict this feeding of powdered diet to specialized applications where powdered diet is absolutely required (e.g., monitoring food consumption in metabolic cages; see Basic Protocol 4 of UNIT 29B.1).

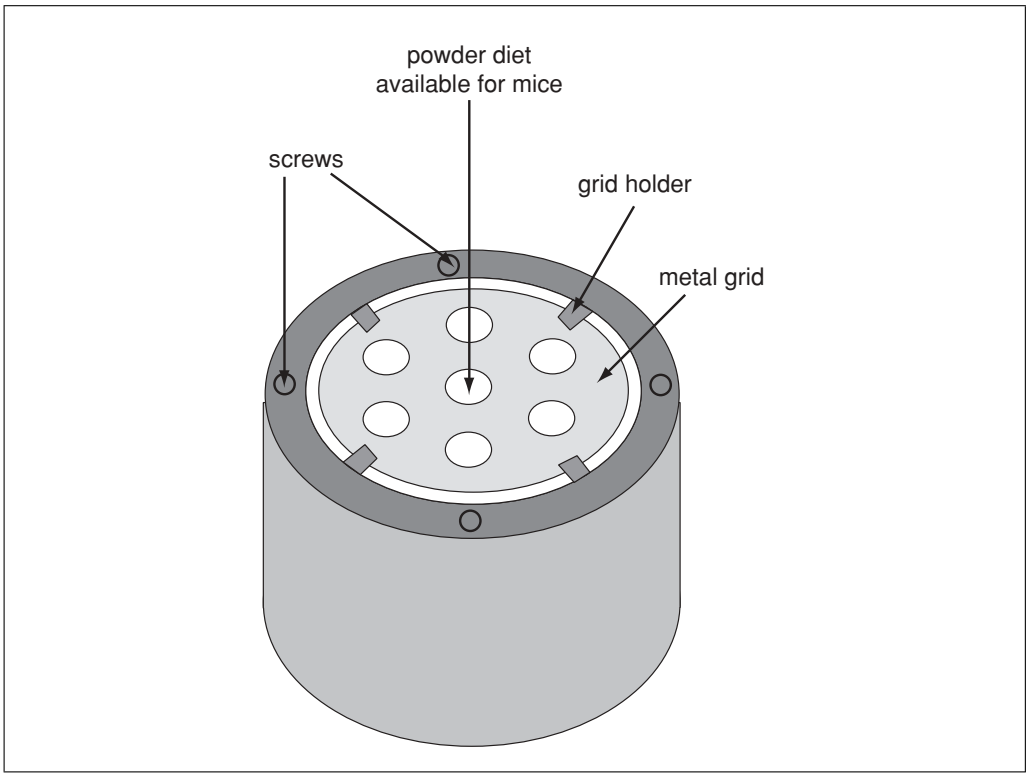

Figure 29B.5.1 Example of a food trough for powdered diet. Powdered diet is placed in the barrel-shaped container and the metal grid (which limits food spoiling and contamination) is positioned on top of the diet and secured with screws and a grid holder. Mice access food via the small holes of the grid.

BASIC PROTOCOL 3

CALORIC RESTRICTION

Caloric restriction (CR) is the main nongenetic manipulation known to extend lifespan in a wide variety of animal models. Although various CR strategies have been experimentally tested, with different levels of feeding restriction, CR is typically defined as a 33% to 50% decrease in total caloric intake. In order to maintain a balanced nutritional supply, diet composition is unchanged to ensure that all sources of nutrients, vitamins, and minerals are provided, and only the quantity of food the animals can access daily is held at levels lower than ad libitum.

The altered metabolic rate induced by this dietary manipulation has been demonstrated to produce a metabolic profile desirable for treating diseases of aging in both animal models and humans (e.g., metabolic disorders or neurodegeneration; Koubova and Guarente, 2003; Masoro, 2005). CR has also been shown to reduce the incidence and progression of spontaneous and induced tumors (Weindruch and Walford, 1988). It is thus not surprising that caloric-restricted mice are a model used extensively to understand the fundamental mechanisms underlying these prevalent human pathologies, and that developing pharmacological caloric restriction mimetics is a current research strategy for antineurodegenerative and anticancer interventions (Roth et al., 2005; Elliott and Jirousek, 2008).

An easily applicable method for restricting caloric intake is to feed the CR group with the same diet as a control group fed ad libitum, but to fast them for 24 hr on alternate days. This method leads to a 30% to 40% decrease of the global caloric intake (Weindruch et al., 1986). However, it generates cycles of lipolysis and fat storage that are not representative of a constant reduction in caloric intake.

The most reliable method for restricting caloric intake consists of pair-feeding CR mice at 50% to 67% of a control group fed ad libitum by restraining their daily food intake. A caloric restriction of 40% is recommended (i.e., pair-feeding at 60% of the ad libitum control). Ideally, mice should be housed in individual cages. If this is not possible, they should at least be housed at the same number of animals per cage. The quantity of food to be administered to the CR group should be evaluated daily as described in the steps below.

Materials

Mice (see Commentary)
Appropriate diet

Housing cages (individual housing, recommended)
Scale with 0.1 g accuracy (to weigh mice and food)
Container to accommodate mice during weighing

1. Monitor daily the food intake (FI) of the control (C) group, fed ad libitum, as described in Basic Protocol 2.
2. On day *n*, at a given hour of the day, change mice of both the C and the caloric restriction (CR) groups to clean cages. Feed the C group ad libitum and adjust the quantity of food administered to the CR group based on the FI of the C group on the preceding day and the percentage of restriction desired (generally 40%).
3. Calculate the quantity of food administered to the CR group on any given day ($QFA_{CRd\mathit{n}}$) in g/cage/day as follows:

$$QFA_{CRd\mathit{n}} = \frac{(100 - PRD)FI_{Cd\mathit{n}-1}}{100} n_m$$

Equation 29B.5.3

where PRD is the percentage of restriction desired;

$FI_{Cdn\text{-}1}$ is the food intake of the control group on the preceding day in g/mouse/day;

n_m is the number of mice per cage.

4. Repeat the FI measurements and the pair-feeding of the C and CR group daily at a constant hour of the day.

COMMENTARY

Background Information

Obesity is a multifactorial disorder that results from an imbalance between caloric intake and expenditure, where the excess of energy is stored as triglycerides, primarily in the adipose tissue. Under such circumstances, triglycerides also accumulate in peripheral tissues (e.g., the liver or the muscle) and exert deleterious consequences on insulin signaling, which leads to a broader pathology where glucose homeostasis is also altered. The C57BL/6 mouse strain is prone to develop obesity when challenged by a diet enriched in fat. Typical high-fat diets contain between 40% and 60% of energy as fat (Table 29B.5.1), and the ability of a diet to promote weight gain directly correlates with its fat content (Fig. 29B.5.2).

As explained above, the action of high-fat and very-high-fat diets on obesity will consequently lead to insulin resistance and ultimately hyperglycemia (Surwit et al., 1988), although it is important to note that several mouse strains are partially resistant to obesity-induced hyperglycemia (Surwit et al., 1988). Alternately, a wider metabolic impact with a more striking hyperglycemic phenotype can be achieved by combining a high-fat with a high-sucrose (or other simple carbohydrates such as fructose) content in the diet (Surwit et al., 1995). This combination is often referred to

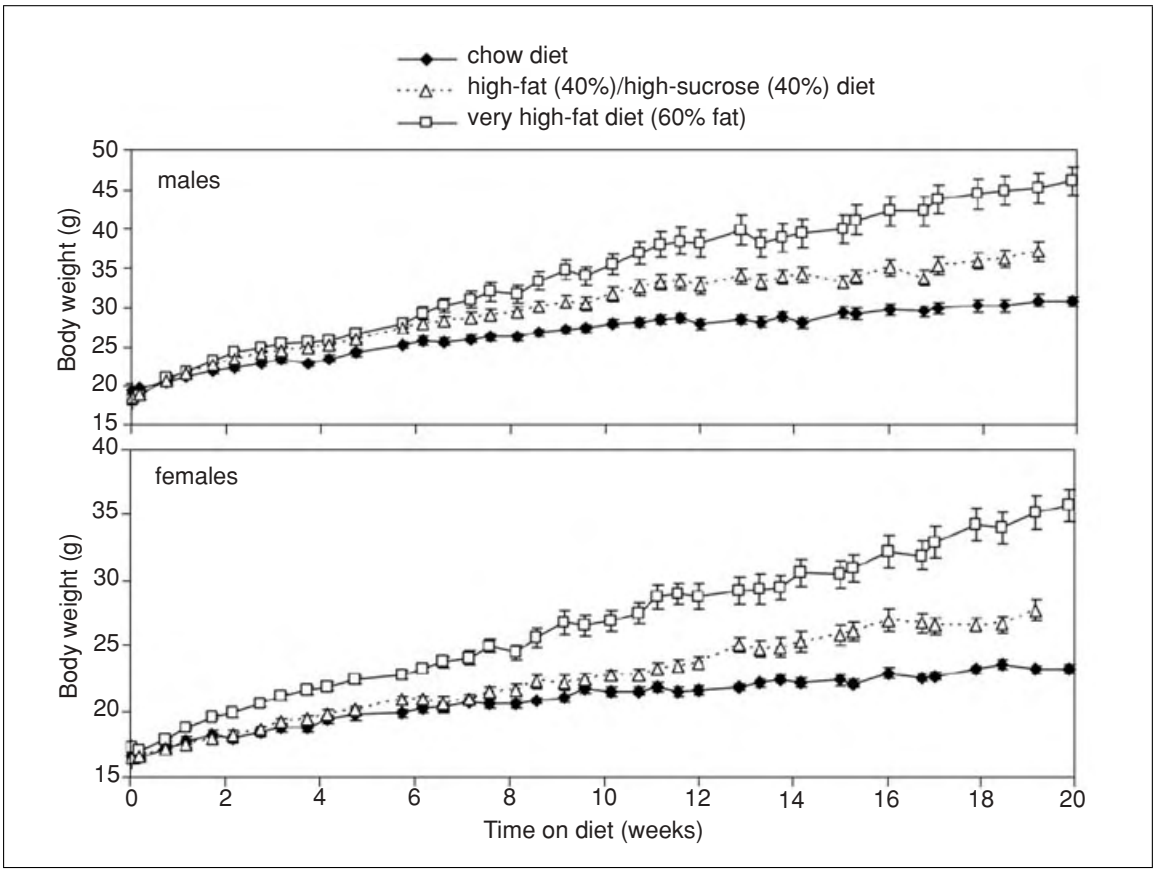

Figure 29B.5.2 Effects of chow and high-fat diets on body weight. Three groups of 7-week male and three groups of 7-week female C57BL/6J mice were housed at five animals per cage and fed ad libitum with chow diet (D04, Safe Diets), high-fat/high-sucrose diet (40% of energy as fat and 40% as sucrose; D12327, Research Diets), or very-high-fat diet (60% energy as fat; D12492, Research Diets). Body weight was recorded twice weekly over twenty weeks on ten animals per group.

as a "western diet" because of its similarity to lipid- and sugar-rich fast-food meals, one of the causes of the increasing prevalence of metabolic diseases in western societies.

Ketoacidosis is an acidification of the plasma resulting from an excessive production of acidic ketone bodies (acetoacetate, β-hydroxybutyrate, and acetone) when cells use fatty acids preferentially over glucose. This pathology can arise when glucose utilization is impaired (e.g., in diabetic patients with a higher prevalence in type 1 as opposed to type 2, diabetics). Features recapitulating this pathology can be induced in mice by totally removing carbohydrates from the diet. Calories are then provided by elevating the fat content, which is the primary source of ketone bodies, but also possibly by enhancing protein concentrations, as amino acid deamination also leads to ketone body production.

Another facet of metabolic research relates to cholesterol homeostasis, as hypercholesterolemia is a major cause of atherosclerosis and its cardiovascular complications. Mice are not a representative model of human cholesterol homeostasis because, unlike in humans, cholesterol is predominantly associated with high-density lipoproteins and can be cleared more efficiently from the bloodstream through conversion to bile acids and subsequent intestinal elimination. Since standard rodent diets are generally poor in cholesterol, high-cholesterol diets are necessary to study cholesterol homeostasis in a setting more relevant to human physiology.

Hypertension is a parameter often correlated with obesity and insulin resistance, and it contributes to the accelerated development of cardiovascular complications associated with metabolic disorders. However, hypertension can be induced in the absence of other metabolic abnormalities by using high-sodium diets and/or chemical models such as mineralocorticoids or nitric oxide synthase inhibitors.

Critical Parameters

Animal housing

The number of animals per cage is a critical parameter in dietary intervention studies. Housing in individual cages is advantageous in certain situations where dominant behavior can be problematic. For example, the precision of food intake measurements and test compound dosing will benefit from individual housing. Moreover, individual housing is particularly recommended for pair-feeding experiments to induce caloric restriction, as a dominant animal can otherwise eat ad libitum and restrain the food intake of its cage mates to levels lower than those desired. In the choice of individual housing, it should be kept in mind that mice are social animals, for whom isolated housing can lead to elevated levels of stress. When collective housing is chosen, the number of animals per cage should always be matched for all the cages of the experiment, as density can profoundly affect behavior, feeding habits, and metabolic homeostasis (Champy et al., 2004). Depending on cage size, three to five animals per cage is generally considered optimal.

Number of animals per group

Dietary manipulations, like many metabolic experiments, display high levels of interindividual variability, essentially because they involve a systemic response of the organism that involves a complex inter-organ cross-talk. Strictly controlled animal housing and experimental conditions are definitely important parameters to limit this variability. Nevertheless, a minimum of eight animals per group is recommended to delineate subtle biological variations from background variability.

Chow diet composition

Given the wide diversity of animal food suppliers, the composition of chow diet can vary slightly according to individual research and development optimization. Changes in chow diet supplier should therefore be minimized, and the composition and batch reference of a diet should be archived, as it constitutes an important experimental parameter. In addition, chow diets have only a partially defined composition, as their main constituents are agricultural cereals of complex composition, which allow relatively inexpensive production costs.

While the global nutritional content is generally well controlled, some specific variations may arise at similar crude composition depending on the source of the primary products. For example, phytoestrogen content can vary greatly depending on the proportion and the source of soy (Thigpen et al., 2004). Consequently, the use of chow diet as a reference for direct comparison with the experimental diets listed in Table 29B.5.1, which are most often formulated according to different and better-defined compositions, should be considered only with these limitations in mind, and diet compositions should be stated as an integral part of any experimental procedure (Warden and Fisler, 2008).

The composition of chow diet indicated in Basic Protocol 1 can be slightly modulated according to the special needs of the animals due to their growing phase. For instance, growing, breeding, and nursing mice, as well as some sensitive mouse strains, can benefit from a chow diet containing ~3500 kcal/kg, in which protein and lipid levels represent about 23% and 5.5% of caloric intake, respectively, and mineral and vitamin content is consequently optimized.

Induction of metabolic disorders

It should be noted that the nature of the fat contained in the diet can considerably impact weight gain and metabolic alterations, according to the proportion of saturated to unsaturated fat and the composition in fatty acids. In that respect, high-fat diet made from animal fat is a widely used challenge that will generally give the strongest metabolic alterations because of the high saturated fat content (Buettner et al., 2007). Context-specific considerations may, however, lead to the use of alternate diets made from vegetable oils chosen according to their fatty acid composition. In any case, the action of a high-fat diet on weight gain will give rise, even on a pure genetic background, to some inter-individual variability, with some mice being very sensitive while others are resistant.

Even on a pure genetic background, the strain of mice is an important parameter to consider for the induction of diet-induced metabolic disorders. 129SvPas mice, commonly used for gene targeting strategies, are resistant to age-induced obesity and insulin resistance (Champy et al., 2008). In contrast, C57BL/6 mice are more susceptible to both diet-induced obesity and hyperglycemia (Surwit et al., 1988), the latter of which has recently been attributed to a reduced capacity to secrete insulin due to a mutation of the

nicotinamide nucleotide dehydrogenase, an enzyme controlling mitochondrial metabolism in pancreatic β cells (Freeman et al., 2006).

It should be kept in mind, however, that high-fat diet–induced hyperglycemia occurs over relatively long periods in most mouse strains because of the capacity of mouse pancreatic β-cells to maintain high secretory levels of insulin despite peripheral insulin resistance. When using high-fat diet to induce global metabolic disorders, it is therefore important to select diets with a very high fat content (60% of energy supplies) and to plan long treatments (>10 weeks) in order to induce both obesity and its negative consequences on glucose homeostasis. A wider metabolic impact with a more striking hyperglycemic phenotype can be achieved by combining high-fat and high-sucrose content in the diet (Surwit et al., 1995). It should be noted, however, that the use of high-sucrose diet alone is rather inefficient at impairing glucose homeostasis in mice. Ketogenic diets lead to weight loss in mice as they strongly stimulate fat oxidation.

High-cholesterol diets are not sufficient to promote cholesterol-related diseases such as atherosclerosis because of the resistance of wild-type mice to hypercholesterolemia. A classical way of overcoming this resistance is to combine high cholesterol with high cholic acid in the diet, because this bile acid promotes uptake of dietary cholesterol and inhibits the rate limiting enzyme that catalyzes the conversion of cholesterol to bile acids through a feed-back loop, thereby reducing its clearance (Houten et al., 2006). In addition, atherogenic lesions can be amplified by combining elevated fat content with high cholesterol and cholic acid levels in a diet generally referred to as the Paigen diet (Paigen et al., 1985, 1987; Nishina et al., 1990). Alternatively, a high-cholesterol diet alone can be efficient in genetic models predisposed to atherosclerosis, such as apolipoprotein E (apoE) or low-density lipoprotein (LDL) knock-out mice.

Mice are not very sensitive to diet-induced hypertension. For example, C57BL/6 mice exhibit elevated blood pressure only after several months of feeding with a diet high in NaCl. The use of high-salt diets in combination with surgical (nephrectomy) and chemical (mineralocorticoids such as deoxycorticosterone acetate—DOCA) models therefore proves more useful for rodent models of hypertension. In addition, hypertension can also be induced by salt-independent procedures such as administration of the nitric oxide synthase inhibitor *Nω*-nitro-L-arginine-methyl ester (L-NAME).

Incorporation of a test compound in the diet

Ensuring homogenous incorporation of the compound in the diet is key to constant dosing of the animals. The method of incorporating the compound differs according to the amount of compound used in mixing and its chemical nature (Fig. 29B.5.3). If the QCD value exceeds 100 mg compound/kg diet for incorporation in chow diet, or 500 mg compound/kg diet for incorporation in high-fat diet, the compound can be directly mixed into powder diet. Grinding of the compound may prove necessary in some cases to ensure homogenous incorporation.

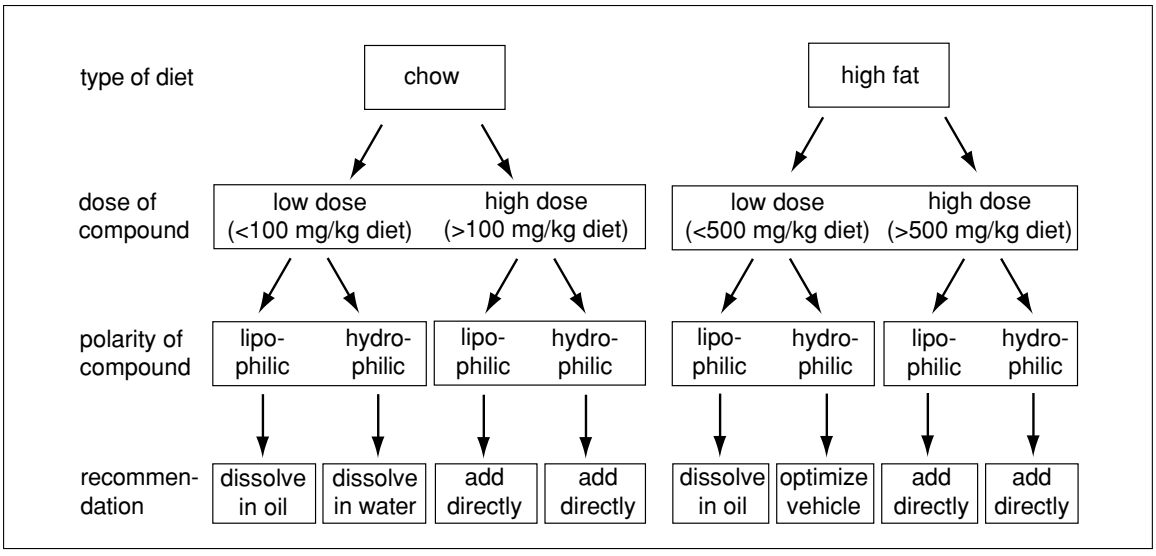

Figure 29B.5.3 Decisional flowchart for test compound incorporation in a diet.

If the QCD values are lower than indicated above, the compound should be dissolved in a vehicle adapted to its polarity to ensure homogenous incorporation to the powder diet. When a hydrophilic compound is to be incorporated in chow diet, water is the vehicle of choice at a volume of 600 ml/kg diet (then replacing step 6b of Basic Protocol 2), but the vehicle and its concentration need to be optimized according to polarity when a hydrophilic compound is to be incorporated in high-fat diet. In contrast, when a hydrophobic compound is to be incorporated in either chow or high-fat diet, oil becomes the best vehicle and should be added to the diet at 10 ml/kg. However, the oil used as vehicle can increase the caloric density of the diet and should be taken into account when analyzing the results.

Administration of compounds at a constant concentration over the entire treatment with high-fat diets is not recommended, as the dosing normalized to body weight will vary during treatment because of weight gain. Similarly, administration at a constant concentration should be avoided when the compound itself affects body weight or feeding behavior.

Diet preservation

While regular chow diet can be stored at room temperature for several months, the elevation of fat content in diets used for metabolic intervention render these diets much more perishable. All diets with a high-fat content should be stored at 4°C over a short term (<2 months) and frozen for longer preservation, according to the manufacturer's recommendations. When a test compound is incorporated in the diet, the time and conditions of storage should be adapted to the stability of the compound.

Caloric restriction

It is best to start CR at 6 weeks of age to avoid any developmental defects.

Anticipated results

Diet-induced metabolic disorders

The actions of very-high-fat and high-fat/high-sucrose diets on body weight are depicted in Figure 29B.5.2.

Caloric restriction

CR can promote an extension of lifespan by up to 50% in rodent models (Koubova and Guarente, 2003), but lifespan extension can vary according to the genetic background, with C57BL/6 mice being more responsive than DBA mice (Ferguson et al., 2007). In addition, CR will prevent weight gain without inducing weight loss in lean adult mice (Ferguson et al., 2007).

Time Considerations

The impacts of dietary manipulations are typically both time dependent and progressive. Typical time frames for inducing different pathologies in the C57BL/6 background are indicated in Table 29B.5.1. However, the duration of the dietary intervention must be adapted to the degree of pathological severity pursued, according to the literature and to the experimenter's experience. For example, a high-fat diet will induce mild glucose intolerance after only 4 weeks, but this intolerance will gradually increase with time and the final symptoms of type 2 diabetes (fasting hyperglycemia, pancreatic dysfunction) will only be observed at later times, after at least 10 weeks on the diet. In addition, the time course of the impact of diet on different strains or on genetically manipulated animals can vary greatly and must be evaluated based on the literature and personal observations.

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Jérôme N. Feige and Marie Lagouge contributed equally to this work.

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CHAPTER 29
Mouse Phenotyping

INTRODUCTION

When *Current Protocols in Molecular Biology* first appeared in 1987, cloning and sequencing mammalian genes were major goals of many laboratories. That era of gene discovery ended with the completion of the genome sequences for humans and increasing numbers of mammalian and other species. From a broad perspective, the determination of these genome sequences is a remarkable accomplishment of which molecular biologists can be proud. However, they are not just trophies to be put on the shelf or files to be stored on hard drives, but rather some of the most powerful tools available in the current era of gene function.

Exploiting these tools is dependent on appropriate biological systems. "Simple" model organisms such as *Drosophila* and *C. elegans* have provided major insights into developmental processes. Other problems, such as metabolic diseases and complex behaviors, are best addressed in mammalian species. Decades of physiologic, endocrinologic, and behavioral studies have focused on rodents, and this deep pool of information is now complemented by extensive genetic tools. Modern genetic studies in mice, like *Drosophila*, date to the pioneering work of Haldane (Haldane, J.B.S., Sprunt, A.D., and Haldane, N.M. 1915. Reduplication in mice. *J. Genet.* 5:133-135.5). As described in Chapter 23, however, classic genetic approaches have been supplemented by powerful techniques for manipulation of the mouse genome. These extensive tools make the laboratory mouse, *Mus musculus*, the mammalian system of choice for genetic analysis.

These diverse genetic technologies produce large numbers of mice with potential phenotypes of all types. In some cases, loss of gene function results in prenatal lethality. This is perhaps the most obvious phenotype and highlights the potential importance of the gene product. It can also be problematic because it substantially complicates detailed analysis of molecular mechanisms. In many other cases, the phenotypes of gene knockouts are less severe than anticipated, often due to functional redundancy. In such circumstances, more detailed analysis often reveals strong phenotypic effects that are not immediately evident. This chapter will present a variety of approaches to analyze phenotypes of mutant mice, with an initial focus on metabolic parameters.

UNIT 29A.1 reviews approaches to genetic manipulation in the mouse, including both knockout and transgenic technologies detailed in Chapter 23 and modern approaches to analysis of quantitative traits using inbred strains. General issues faced in mouse phenotyping, such as housing and other environmental factors, are considered in *UNIT 29A.2*. *UNIT 29A.3* addresses some specific procedures needed for metabolic phenotyping, specfically collection of blood, plasma, and serum. The broader issue of examining and collecting mouse tissues is covered in *UNIT 29A.4*, which provides a comprehensive procedure for post-mortem analysis of anatomical defects (necropsy), as well as a systematic approach to tissue collection for both histology and molecular biology applications.

UNIT 29B.1 begins the section on metabolic regulation with analysis of energy balance. Because body weight is so tightly regulated at a variety of levels, it is surprising that many mutant mice exhibit either increased or decreased weight gain, particularly in response to high fat, high sucrose, or "Western" diets. *UNIT 29B.5* describes the implementation of

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such dietary manipulations, and *UNIT 29B.1* presents a detailed plan for analysis of body weight, composition, and energy balance. Because body weight is so tightly regulated at a variety of levels, it is surprising that many mutant mice exhibit either increased or decreased weight gain, particularly in response to high-fat diets or other dietary challenges. Such changes are often associated with dysregulation of lipid and/or glucose homeostasis. *UNIT 29B.1* presents a detailed plan for analysis of such phenotypes. *UNIT 29B.2* addresses the analysis of both serum and tissue levels of lipids, including cholesterol and its downstream metabolites, bile acids, and free or non-esterified fatty acids (FFA; NEFA) and triglycerides (TG). Fractionation methods for determination of lipoprotein profiles are also included. *UNIT 29B.3* focuses on glucose homeostasis. Extensive protocols for analysis of serum glucose and insulin are provided, including relatively standard glucose and insulin tolerance tests as well as the "gold standard" hyperinsulinemic-euglycemic clamp. For all of these metabolic units, extensive discussion of critical parameters and anticipated results facilitates navigation of this complex area.

Histologic analysis of cells and tissues is another key aspect of mouse phenotyping. *UNIT 29B.4* describes an extensive array of histopathologic techniques, with a particular focus on post-mortem metabolic studies. Basic principles of routine histology including tissue fixation and staining with hematoxylin and eosin are followed by several staining protocols commonly used in the metabolic field, such as stains for intracellular lipids, cholesterol esters, and polysaccharides. These are supplemented by specific procedures for detection of adipophilin to identify intracellular lipid "ghosts," for detection of succinate dehydrogenase and mitochondria in muscle tissue, for sirius red staining of pathological collagen deposits, and en face staining of atherosclerotic plaques in aortas with sudan IV.

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